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FIBROBLAST GROWTH FACTOR 8 AND ITS RECEPTORS IN THE GROWTH AND ANGIOGENESIS OF EXPERIMENTAL BREAST CANCER

with Special Reference to Thrombospondin-1 as a Novel Target Gene for FGF-8

by

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To Emma and Anna

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ABSTRACT

The growth of breast cancer is regulated by hormones and growth factors. Recently, aberrant fibroblast growth factor (FGF) signalling has been strongly implicated in promoting the progression of breast cancer and is thought to have a role in the development of endocrine resistant disease. FGFs mediate their auto- and paracrine signals through binding to FGF receptors 1-4 (FGFR1-4) and their isoforms. Specific targets of FGFs in breast cancer cells and the differential role of FGFRs, however, are poorly described. FGF-8 is expressed at elevated levels in breast cancer, and it has been shown to act as an angiogenic, growth promoting factor in experimental models of breast cancer. Furthermore, it plays an important role in mediating androgen effects in prostate cancer and in some breast cancer cell lines. We aimed to study testosterone (Te) and FGF-8 regulated genes in Shionogi 115 (S115) breast cancer cells, characterise FGF-8 activated intracellular signalling pathways and clarify the role of FGFR1, -2 and -3 in these cells. Thrombospondin-1 (TSP-1), an endogenous inhibitor of angiogenesis, was recognised as a Te and FGF-8 regulated gene. Te repression of TSP-1 was androgen receptor (AR)-dependent. It required *de novo* protein synthesis, but it was independent of FGF-8 expression. FGF-8, in turn, downregulated TSP-1 transcription by activating the ERK and PI3K pathways, and the effect could be reversed by specific kinase inhibitors. Differential FGFR1-3 action was studied by silencing each receptor by shRNA expression in S115 cells. FGFR1 expression was a prerequisite for the growth of S115 tumours, whereas FGFR2 expression alone was not able to promote tumour growth. High FGFR1 expression led to a growth advantage that was associated with strong ERK activation, increased angiogenesis and reduced apoptosis, and all of these effects could be reversed by an FGFR inhibitor. Taken together, the results of this thesis show that FGF-8 and FGFRs contribute strongly to the regulation of the growth and angiogenesis of experimental breast cancer and support the evidence for FGF-FGFR signalling as one of the major players in breast cancers.

Keywords: FGF-8, FGFR, angiogenesis, breast cancer, TSP-1, androgen

Kati Tarkkonen

FIBROBLASTIKASVUTEKIJÄ 8 JA SEN RESEPTORIT KOKEELLISEN RINTASYÖPÄMALLIN KASVUSSA JA ANGIOGENEESISSÄ
Trombospondiini-1 FGF-8:n kohdegeeninä

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TIIVISTELMÄ

Hormonit ja kasvutekijät säätelevät rintasyövän kasvua. Viimeaikaiset tutkimustulokset osoittavat, että häiriintynyt fibroblastikasvutekijä (FGF) signalointi edistää rintasyövän etenemistä ja on osallisena hormoneille vastustuskykyisen tautimuodon kehittymisessä. FGF:t toimivat välittämällä auto- ja parakriinisia vaikutuksia sitoutumalla solukalvon FGF reseptoreihin (FGFR1-4). Niiden erityiset kohteet rintasyöpäsoluissa ovat kuitenkin toistaiseksi suurelta osin tuntemattomia. FGF-8 ilmentyminen on kohonnut rintasyövissä, ja sen on osoitettu toimivan verisuonten uudismuodostusta eli angiogeneesiä sekä kasvua lisäävänä tekijänä rintasyövän kokeellisissa malleissa. Lisäksi sen on osoitettu välittävän androgeenien vaikutuksia eturauhassyövässä sekä joissain rintasyöpäsolulinjoissa. Tämän tutkimuksen tavoitteena oli tunnistaa testosteronin (Te) ja FGF-8:n säätelemiä geenejä Shionogi 115 (S115) rintasyöpäsoluissa, tutkia FGF-8:n aktivoimia solunsisäisiä signaalinsiirtoreittejä, sekä selvittää FGFR1, -2 ja -3 roolia näiden solujen kasvussa. Totesimme Te:n ja FGF-8:n vähentävän angiogeneesiä estävän geenin, thrombospondiini-1:n (TSP-1), ilmentymistä. Lisätutkimukset osoittivat, että Te ja FGF-8 vaikutukset ovat toisistaan riippumattomia: Te aiheutti TSP-1 tason laskun androgeenireseptori (AR) kautta, kun taas FGF-8 vähensi TSP-1 transkriptiota aktivoimalla ERK ja PI3K proteiinikinaasireittejä. FGFR1-3 toisistaan eroavia vaikutuksia tutkittiin hiljentämällä niiden ilmentymistä S115 soluissa shRNA-tekniikkaa käyttäen. Osoitimme, että FGFR1 on välttämätön S115 solujen kasvulle kateenkorvattomissa nude-hiirissä, kun taas FGFR2 yksin ei pystynyt saamaan aikaan tuumorien kasvua. Korkea FGFR1 taso johti S115 soluissa voimakkaampaan ERK aktivaatioon, kohonneeseen angiogeenisyyteen ja ohjatun solukuoleman eli apoptoosin vähenemiseen. Kaikki nämä vaikutukset pystyttiin kumoamaan estämällä FGFR:ien toiminta FGFR-inhibiittorilla. Yhteenvetona voidaan todeta, että tulokset osoittivat FGF-8:n ja FGFR:ien olevan tärkeitä tekijöitä kasvaimen kasvun ja angiogeneesin säätelyssä kokeellisessa rintasyöpämallissa, ja siitä johtuen tämä väitöskirja edelleen vahvistavaa käsitystä siitä, että FGF-FGFR signalointi voi olla tärkeässä osassa rintasyöpien kasvua.

Avainsanat: FGF-8, FGFR, angiogeneesi, rintasyöpä, TSP-1, androgeeni

TABLE OF CONTENTS

ABBREVIATIONS

AR	androgen receptor
CAM	chorion allantoic membrane assay
CGH	comparative genomic hybridization
CHX	cycloheximide
CM	conditioned medium
COX	cyclo-ozygenase
CSC	cancer stem cell
CSF-1	colony stimulating factor-1
DAG	diacylglycerol
DC	dextran charcoal
DHT	dihydrotestosterone
DMEM	Dulbeccos modified eagle medium
E2	estradiol
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial to mesencymal transition
ER	oestrogen receptor
ERE	oestrogen responsive element
ERK	extracellular signal related kinase
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FGFRL	fibroblast growth factor like
FRS2	fibroblast growth factor receptor substrate 2
GH	growth hormone
Grb2	growth factor receptor bound 2
HE	hematoxylin and eosin
Her2	human epidermal growth factor receptor 2
HIF	hypoxia inducible factor
HisH3	histone H3
HRP	horseradish peroxidase
HRT	hormone replacement therapy
HSPG	heparin sulphate protein glycan
iFBS	heat inactivated fetal bovine serum
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IR	insulin receptor
JNK	c-Jun N-terminal kinase
KO	knockout
LOH	loss of heterozygosity
MAPK	mitogen activated protein kinase
MKP	mitogen activated kinase phosphatase
MMP	matrix metalloproteinase
MMTV	mouse mammary tumour virus
NCAM	neural cell adhesion molecule
OPN	osteopontin
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
IP3	inositol 1,4,5-triphosphate
PI3K	phostphatidyl inositol-3 kinase
PlGF	placental growth factor
PR	progesterone receptor
PTB	phosphotyrosine binding

qPCR	quantitative polymerase chain reaction
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase polymerase chain reaction
sc	subcutaneous
Sef	similar expression to FGF
SERM	selective oestrogen receptor modulator
SH2	Src homology 2
shRNA	short hairpin ribonucleid acid
SNP	single nucleotide polymorphism
SOS	son of sevenless
STAT	signal transducer and activator
Te	testosterone
Tam	tamoxifene
TBS	tris buffered saline
TDLU	terminal duct lobular unit
TEB	terminal end bud
TGF	transforming growth factor
TK	tyrosine kinase
TKI	tyrosine kinase inhibitor
TNBC	triple negative breast cancer
TSP-1	thrombospondin-1
TSR	type three repeat
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by the Roman numerals I-III:

I Mirjami Mattila*, Kati Tarkkonen*, Jani Seppänen, Johanna Ruohola, Eeva Valve and Pirkko Härkönen (2006): Androgen and fibroblast growth factor 8 (FGF8) downregulation of thrombospondin 1 (TSP-1) in mouse breast cancer cells. Mol Cell Endocrinol. 253(1-2):36-43. *equal contribution

II Kati Tarkkonen, Johanna Ruohola and Pirkko Härkönen (2010) Thrombospondin-1 (TSP-1) is downregulated by finbroblast growth factor 8 (FGF8) through activation of ERK pathway. Growth Factors, 28(4):256-267.

III Kati M. Tarkkonen*, Emeli M. Nilsson*, Julien H. Dey, Jari E. Heikkilä, Tiina E. Silvola, Johanna M. Tuomela, Qing Liu, Nancy E. Hynes and Pirkko L. Härkönen. Differential role of fibroblast growth factor receptor (FGFR) 1, 2 and 3 in regulation of S115 breast cancer cell growth. Submitted manuscript. *equal contribution

1 INTRODUCTION

Breast cancer is the most common cancer in females. Most breast cancers are originally oestrogen dependent and response to inhibition of oestrogen receptor (ER) function, but eventually many patients with localised disease and all the patients with metastatic disease become resistant to endocrine therapy. The cause for the development of resistance is partly explained by the alterations in ER signalling, such as ligand independent signalling and increased sensitivity for oestrogens, but in addition, the involvement of growth factor signalling has become evident in the hormone-independent growth. Even in normal breast tissue, the hormone action is largely mediated by the regulation of paracrine acting growth factors, such as the TGFβ, EGF, IGF and FGF growth factor families. Thus, it is apparent that the dysregulation of growth factor signalling may be a major contributor in the progression of breast cancer to metastatic and endocrine-resistant disease.

Fibroblast growth factors (FGFs) belong to a growth factor family that plays an important role during embryogenesis and tissue homeostasis. FGFs mediate their effects through cell membrane tyrosine kinase receptors (FGFR1-4), which exist in several isoforms. Although FGFR1-4 have overlapping expression patterns and functional similarities, they also mediate very specific effects depending on the cellular stage and context (Dailey *et al.*, 2005). During the past decades, FGFs have been shown to play a crucial role in the malignant transformation and proliferation of breast cancer cells as well as in tumour angiogenesis in experimental models of breast cancer. Recently, increasing evidence shows that FGF-FGFR signalling is an important contributor to the progression of human breast cancer. For example, genetic alterations of several FGFR forms associated with human breast cancer have been discovered. Such alterations include the amplification and over-expression of FGFR1 (Reis-Filho *et al.*, 2006; Turner *et al.*, 2010b), single nucleotide polymorphisms (SNP) in the FGFR2 gene resulting in an increased risk for breast cancer (Easton *et al.*, 2007; Hunter *et al.*, 2007), and a SNP in FGFR4 gene that is linked to drug resistance in breast cancer (Meijer *et al.*, 2008; Marme *et al.*, 2010b). FGF-8 is one of the few FGFs that are expressed at elevated levels in human breast cancer (Marsh *et al.*, 1999). It has been shown to mediate proliferative, angiogenic, and antiapoptotic responses in experimental models of breast cancer (Mattila and Harkonen, 2007). Although the principles of FGF signalling are well documented, the specific target genes and mechanisms of action of FGF-8 are largely unknown. Due to the emerging data on FGFR signalling and its importance in breast and in other human cancers, there is increased interest in developing of small molecule FGFR inhibitors as therapeutic agents. Therefore, recognising the specific targets of certain FGFs and clarifying the differences between different FGFR forms in certain types of cancers is of high importance. This study aims to clarify FGF-8 mediated effects in breast cancer cells and investigate the role of different FGFRs in breast cancer cell proliferation and growth *in vitro* and *in vivo*.

2 REVIEW OF THE LITERATURE

2.1 BREAST CANCER

It is estimated that more than one million women are diagnosed with breast cancer worldwide every year and more than 400 000 will die from the disease (Coughlin and Ekwueme, 2009). In European countries breast cancer is the most commonly diagnosed cancer, with over 4000 new cases each year in Finland (www.cancer.fi/syöpärekisteri). Although the incidence of breast cancer is increasing, the mortality rate has been declining over the past 15 years as a result of screening programs, better education and more effective adjuvant treatments (Peto *et al.*, 2000; Berry *et al.*, 2005; Ferlay *et al.*, 2007). However in developing countries where the disease is often diagnosed in a late stage, the mortally rate is high. Moreover, the most severe concern regarding breast cancer is that 25-40% of patients with breast cancer eventually develop metastatic disease that is largely incurable (Guarneri and Conte, 2004).

2.1.1 Aetiology and origin of breast cancer

Both genetic and lifestyle/environmental factors are implicated in the aetiology of breast cancer. The best known risk factors for breast cancer in addition to age are exposure to oestrogens (Parkin *et al.*, 2001) and a family history of breast cancers (Kumar *et al*., 2005). Other factors such as alcohol use, geographic location, height, higher socio-economic status and obesity contribute also to increased breast cancer risk (Dumitrescu and Cotarla, 2005). In general, breast cancers can be divided into sporadic cases and into hereditary cases that are associated with family history or germ-line mutations. It is intriguing that although family history is considered the major risk factor for the development of breast cancer, only 13% of all women diagnosed with breast cancer have any familial risk factors, and most of women with first-degree relatives with a history of breast cancer, i.e., those who are at increased risk of the disease, will never develop breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer, 2001).

Sporadic breast cancer is thought to result from a serial stepwise accumulation of acquired and uncorrected mutations in somatic genes, without any germ line mutations playing a role. The major risk factors for sporadic breast cancer are related to hormone exposure, mainly to oestrogens, through gender, age at menarche and menopause, reproductive history, breast-feeding history, and exogenous oestrogens. Oestrogens and their metabolites can cause mutations or generate DNA-damaging free radicals, and drive the proliferation of premalignant lesions (Kumar *et al*., 2005). The mechanism of oestrogen action will be described in more detail in later paragraphs. Although oestrogens represent the best known players in the development of breast cancers, the carcinogenesis is a multistep process during which a normal cell must

undergo numerous changes to become malignant and these acquired capabilities can be achieved by several different mechanisms. Thus, there is no common genetic or functional change that can be found in all breast cancers, although some alterations (such as in the case of ER and Her2 function), are more frequent than some others.

The genetic variants associated with breast cancer risk can be further classified as high-penetrance mutations that are rare but are associated with very high risk, moderate penetrance variants that increase the risk moderately, or low-penetrance variants, which are common and are associated with only a small increased risk (Mavaddat *et al.*, 2010). The first identified and the most important high-penetrance susceptibility genes for breast cancer (and for ovarian cancer) are BRCA1 and BRCA2, which were discovered in the early 1990s (Hall *et al.*, 1990; Easton *et al.*, 1993; Wooster *et al.*, 1994). Estimated risks for breast cancer among BRCA mutations carriers are as high as 65% (BRCA1) or 45% (BRCA2) (Antoniou *et al.*, 2003), but there is variation between mutation carriers and families (Mavaddat *et al.*, 2010). Certain variants of genes that carry a moderate risk for breast cancers include, for example, the gene for cell cycle check point kinase CHEK2 (Meijers-Heijboer *et al.*, 2003) and PALB2, which codes for a BRCA1 interacting protein (Rahman *et al.*, 2007). However, the largest and most recently discovered group of genes associated with breast cancer consists of low penetrance polymorphisms, which in combination with one another may contribute strongly to the fraction of "unexplained" familial breast cancers. The sequencing of the human genome and the mapping of single nucleotide polymorphisms (SNPs) in genome-wide association studies of large study populations has led to the identification of twelve susceptibility loci which contain several genes (Mavaddat *et al.*, 2010). For example, genes such as TOX3 and FGFR2 (Easton *et al.*, 2007) are located in or nearby these regions. The role of FGFR2 in breast cancer will be discussed in more detail later in this thesis.

The adult female breast consists of a branched ductal system (parenchyma) surrounded by connective tissue and fatty tissue (mesenchyme or stroma). More than 90% of the mammary parenchymal tissue is composed of two types of differentiated epithelial cells: secretory luminal epithelial cells lining the mammary ducts and alveoli and contractile basal/myoepithelial cells situated in between the luminal cells and the basement membrane (Russo and Russo, 2004). These two epithelial cell populations undergo constant proliferation, differentiation and apoptosis in response to systemic hormones and local growth signals during the menstrual cycle, pregnancy, and aging. The terminal duct lobular units (TDLUs) are are generally considered to be the origin of breast cancer (Russo *et al.*, 2000). During the past decades, there has been much effort put into the characterizing of breast epithelial stem/progenitor cells, which are the cell of origin for the different cell lineages in the breast. In addition, an interesting discussion has been underway regarding the existence of tumour cells with stem cell features, the so-called cancer stem cells (CSCs), in breast cancer. In a recent and extensive review on this research field by Petersen and Polyak, the authors concluded that it is probable that there are actually different stem cells at different stages of development in the breast, and according to the current knowledge, these human

breast epithelial stem cells exist mostly within the basal cell layer. However, the luminal compartment or the reprogrammed luminal cell population can provide multipotent precursors for breast cancer, which explains why most breast cancers display characteristics of this cell lineage (Petersen and Polyak, 2010). Indeed, it has been demonstrated that reprogramming differentiated somatic cells into pluripotent stem cells is possible at least *in vitro* (Park *et al.*, 2008), suggesting that the differentiation program is not as irreversible as was previously thought. Furthermore, there is strong evidence that epithelial to mesenchymal transition (EMT), which is a process by which epithelial cells lose their epithelial cell characteristics to acquire a mesenchymal phenotype and become migratory and invasive, leads to the generation of breast cancer cells with stem cell-like characteristics (Mani *et al.*, 2008; Morel *et al.*, 2008; Blick *et al.*, 2010). However, although the existence of both stem cells and CSCs in the mammary gland is currently widely accepted, there are still numerous open questions regarding their evolution and maintenance in the tissue/tumour microenvironment.

2.1.2 Classification of breast cancers

Breast cancers are traditionally classified based on their histopathology and stage. Most of the breast cancers (95%) are adenocarcinomas, which are further divided into *in situ* carcinomas and invasive carcinomas. The *in situ* carcinomas, which comprise approximately 15-30% of all breast carcinomas, can be classified further into either ductal or lobular carcinomas *in situ* (DCIS or LCIS, respectively), and they may develop into invasive breast cancers (Simpson *et al.*, 2005). In turn, invasive carcinomas include numerous different subtypes such as ductal carcinomas, lobular carcinomas and tubular carcinomas. A number of validated clinicopathological factors are used in the clinic to assess prognosis, guide therapy, and predict the response to therapy. These factors include patient age and menopausal status, tumour type, size and grade, lymphovascular involvement, nodal status, and the status of various markers, such as the oestrogen receptor (ER), progesterone receptor (PR), and EGF receptor Her2 (Morabito *et al.*, 2003).

However, the classical histopathological classification described above does not accurately identify all breast cancers or predict the outcome of individual patients, which has led to the utilisation of new strategies in the attempt to classify breast tumours. The development of methods for the molecular profiling of tumour types, including tissue microdissection, DNA amplification, and genome and transcriptome analysis (such as cytogenetics, comparative genomic hybridisation (CGH), loss of heterozygosity (LOH) analysis, gene expression analysis and microarray CGH) have allowed increasing amounts of data to be collected from breast tumours. However, the interpretation of the large data sets is not simple, and the significance of many findings with respect to previous knowledge is still unclear. According to a microarray-based comprehensive gene expression profiling (GEP) study by Perou *et al.* (2000), breast tumours can be divided into five types: luminal A, luminal B, Her2

positive, basal-like, and normal-like carcinomas. These tumour types correspond reasonably well to clinical characterisation on the basis of ER and Her2 status (Perou *et al.*, 2000; Sorlie *et al.*, 2001). The basal-like or basal phenotype is also sometimes referred to as triple negative (ER-, PR-, Her2-) breast cancers (TNBC) and it is considered to be the most aggressive and difficult to treat breast carcinoma. Recently, however, it has turned out that TNBC and basal-like breast tumours are not synonymes with one another, although it is clear that they share many characteristics (Foulkes *et al.*, 2010). Luminal A type cancers, in turn, which are mostly ER positive and histologically low-grade, are suggested to have the best prognosis. However, this GEP based approach has been criticised because of its simplistic view of the variability of breast cancers. For example, all breast carcinoma subtypes that fulfil the criteria for the basal-like category are not aggressive or they have highly variable behaviour (Rakha *et al.*, 2007; Tavassoli, 2010). Thus, it is evident that more variants of these suggested tumour types will arise as more data are collected, leading to increasing number of molecular variants of breast cancers. One example of a tumour subtype is the "molecular apocrine" breast tumours, which were shown to differ in their gene expression profile from the basal and luminal groups. The name of these ER-negative but AR-positive tumours refers to their increased androgen signalling and some apocrine features (Farmer *et al.*, 2005). Genetic alterations revealed by CGH genomic profiles have shown that there is also a close relationship between the grade of differentiation and the pattern of genomic changes in tumours: thus, CGH arrays can be utilised to identify different forms of breast cancer and to discover new genes involved in breast cancer initiation and progression (Climent *et al.*, 2007). However, in spite of marked advances in the field, additional information and data interpretation are still needed to identify genes and mechanisms that can ultimately be used for improving targeted therapies for certain types of breast cancers.

Importantly, much of the data concerning molecular mechanisms of breast cancer are derived from studies with different breast cancer cell lines. Quite often, a cell line is used without paying any special attention to the particular tumour subtype it represents. However, due to heterogeneity of breast cancers, it would be important to understand which cell line is the best model for phenomenon under study. Recently, molecular profiling of the widely-used breast cancer cell lines has provided new information on the correlation of these cell lines with breast cancer subtypes (Neve *et al.*, 2006; Kao *et al.*, 2009). According to these studies, breast cancer cell lines were clustered into three groups according to their gene expression profiles: luminal, basal-A and basal-B. The results of Kao *et al*. showed that the luminal and basal-A cell lines are the most appropriate models for luminal-B and basal-like cancers, respectively. Grouping based on Her2 over-expression showed some discrepancies between the cell lines and tumours. While most of the Her2 positive cell lines clustered into the luminal group of cell lines, the Her2 positive tumours resembled the gene expression profiles of both luminal and basal-A cell lines. Interestingly, no breast tumour subtype has been identified that would corresponds to the basal-B cell lines, which express many mesenchymal and/or stem cell markers. Furthermore, those basal-B cell lines included non-tumourigenic lines (e.g., MCF10A) and highly invasive lines (e.g., MDA-

MB231). An explanation for the lack of correspondence between the cell lines and tumours was recently reported by Keller *et al*. (2010), who demonstrated that the cellular heterogeneity within both breast cancer cell lines and normal breast epithelial cell lines was remarkably restricted in culture and indeed enriched for cellular phenotypes with basal and mesenchymal phenotypes which are normally present as a minor component within human tissue (Keller *et al.*, 2010). According to this study, any single cell line poorly represents the heterogeneity of breast tumours: thus, it would be recommended to always use a collection of cell lines. A summary of the correlations between the cell line classifications and the tumour subtypes is presented in Table I.

Table I. Relationship of breast cancer cell line types and human tumour subtypes based on gene expression profiling.

TYPE OF THE CELL LINE	**THE MAIN RECEPTOR STATUS OF THE CELL LINES**	**THE BEST CORRESPONDING TUMOUR SUBTYPE**	**EXAMPLES* OF THE CELL LINES**
Luminal	ER+, PR+/-, Her2-/+	Luminal A, B, Her2 positive	T47D, MCF-7, MDA134, BT474, SUM52-PE
Basal A	ER-, PR-, Her2-/+	Basal –like, Her2 positive	MDAMB468, BT20, SUM190BT
Basal B	ER-, PR-, Her-	Not found	MCF10A**, MDA-MB231, SUM159PT

* Examples of the cell lines were chosen randomly from over 50 cell lines included in the original studies.

** MCF10A was recently reported as an ER positive cell line (Yusuf and Frenkel, 2010).

References: (Neve *et al.*, 2006; Kao *et al.*, 2009)

2.1.3 Progression of breast cancer

According to the traditional breast tumour progression model, invasive breast cancers are thought to arise through a stepwise, linear progression from normal breast epithelium to hyperplasia, carcinoma in situ (CIS), and invasive breast cancer as a result of the progressive accumulation of genetic abnormalities in epithelial cells. As discussed in the previous paragraph concerning breast cancer classification, new methods for molecular characterisation and for studying the genomics of breast tumours have provided new insights into breast cancer progression. Furthermore, new information concerning the role of the tumour microenvironment in breast cancer progression has also emphasised the role of players other than the tumour cells themselves in cancer progression (Schnitt, 2010). Thus, the traditional model of breast

cancer progression seems to be too simplistic. However, in spite of the incomplete current knowledge on the precise mechanism of breast cancer progression, it is evident that the majority of breast cancer deaths are due to metastasis to distant organs, such as the lung, bone, brain and liver (Patanaphan *et al.*, 1988; Hess *et al.*, 2006). Processes involved in the development to metastatic breast cancer are described next.

2.1.3.1 Angiogenesis and lymph angiogenesis

Angiogenesis is a prerequisite for the development and growth of solid tumours beyond 1-2 mm^3 (Folkman, 1971). In addition to facilitation of primary tumour growth, angiogenesis enables tumour cells to spread through the bloodstream to distant sites and, thus, to metastasise. Hypoxia is one of the key triggers of the angiogenic switch, a concept that was described by Hanahan and Folkman in 1996 (Hanahan and Folkman, 1996). During the switch to the angiogenic phenotype, the balance of pro- and antiangiogenic factors secreted by tumour or stromal cells is altered, resulting in the rapid growth of new blood vessels. The hypoxic response is primarily regulated by the induction of hypoxia inducible factors (HIFs), which in turn regulate the transcription of several hypoxia-sensitive factors. The overexpression of HIFs correlates with poor prognosis in breast cancer (Schindl *et al.*, 2002). In contrast to the well organized layers of endothelial cells and pericytes in the vessels of normal tissue, tumour-associated vessels are often irregular and unstable (Bergers and Benjamin, 2003). The best known mechanism of vasculature formation in the tumours is endothelial sprouting, but other mechanisms have also been described (Dome *et al.*, 2007). Endothelial sprouting is normally a tightly regulated process in which the balance of pro- and antiangiogenic factors determines the proliferation, migration and tube formation of endothelial cells. Importantly, the angiogenic pathways exhibit a significant degree of cross-activity, which leads to redundancy in the molecular pathways that activate or inhibit angiogenesis (Gordon *et al.*, 2010). Some of the best studied pro- and antiangiogenic factors include VEGF and TSP-1, respectively, and they will be described later in detail.

The lymphatic vasculature is the other route of metastatic spread in cancer. In its normal physiological function, the lymphatic system maintains the homeostasis of tissue fluids by collecting the protein-rich fluid that exudes from the blood vessels and drains it back into the venous circulation through collecting vessels, lymph nodes, lymphatic trunks and ducts (Adams and Alitalo, 2007). During normal development, lymphatic vessels originate from the embryonic veins. Although the endothelia of both vessel types share many common features, lymphatic vessels have a distinct architecture and function from the blood vessels. Importantly, it has been demonstrated that lymphangiogenesis in tumours, i.e., the growth of lymphatic vessels, promotes metastasis via the lymphatics (rev. in Eccles *et al.*, 2007). Like other types of carcinomas, breast cancers have a predilection to initially metastasize to regional lymph nodes (Sleeman, 2000). However, it is not clear whether regional lymph node metastases are only indicators of metastatic progression, or whether they

actually play a crucial role in the systemic dissemination and act by seeding metastatic tumour cells into the blood (Eccles *et al.*, 2007). In theory, there may be several mechanisms of lymphangiogenesis, but observations from animal models suggest that most of the tumour-associated lymphatic vessels are produced by sprouting from pre-existing vessels (He *et al.*, 2004). The key protein that induces lymphangiogenesis is vascular endothelial growth factor receptor-3 (VEGFR-3), which is activated by vascular endothelial growth factor-C and -D (VEGF-C and VEGF-D). Additional factors implicated in lymphangiogenesis include the angiopoietins (Ang-1, Ang-2, and Ang-3), FGF-2, platelet-derived growth factor (PDGF), insulin growth factor-1 and -2 (IGF-1, -2), hepatocyte growth factor (HGF) and growth hormone (GH) (Ran *et al.*, 2010).

2.1.3.2 Metastasis

As mentioned in the introduction, metastatic breast cancers still remain an uncurable disease in most cases. To metastasise, tumour cells must invade locally to the surrounding stroma and pass through physical barriers to enter into the bloodstream (intravasation). They must then survive in the circulation and subsequently adhere to a vessel wall and exit the bloodstream (extravasation), colonise to a distant organ and eventually develop overt metastases (Nguyen *et al.*, 2009). Thus, the metastatic process is complicated, and failure at any of the above mentioned steps can block the entire process. It is important to note that malignant transformation as such is not sufficient for metastatic competence, and a tumour cell must acquire additional abilities required, for example, in infiltration and colonisation to finally develop distant macrometastases (Klein, 2003). Sometimes the infiltrated cancer cells may stay latent for a long period of time by staying in a dormant state, i.e., by proliferating at a rate that is counterbalanced by cell death. In the case of ER-positive breast cancers, it is known that cancer cells may infiltrate to distant sites at early stages but that they frequently enter a prolonged period of latency, which explains the fact that metastases can manifest decades after the removal of the primary tumour (Lee, 1985; Schmidt-Kittler *et al.*, 2003). However, the detailed molecular mechanisms of cancer metastasis and the processes involved are still poorly understood. The development of microarray methods has enabled the identification of gene sets that may indicate the prognosis of breast cancer patients, such as the first reported “poor prognosis signature” of 70 genes including many genes that regulate the cell cycle, invasion, metastasis and angiogenesis (van 't Veer *et al.*, 2002). To date, there are several commercially available prognostic signatures, which can be used to stratify breast cancer patients by tumour types and to predict patients’ responsiveness to specific therapies (Dunn and Demichele, 2009). However, there is still a need for further development and validation of the signatures for this technology to be widely used in the clinics. Specific metastasis-related gene signatures have also been discovered experimentally, for example, by comparing human MDA-MB231 cell line clones with high or low capacity to metastasise to the bone in mouse studies (Kang *et al.*, 2003). Interestingly, the genes found in the bone metastasising clones of MDA-MB231 were not included

in the poor prognosis signature mentioned above, which suggests that the increased expression of tissue-specific metastatic genes may emerge later during the progression of the primary tumour and may also suggest that a given combination or set of genes may be utilised by only a fraction of breast tumours that metastasise to bone (Kang *et al.*, 2003). In addition to bone, a specific set of genes involved in the passage of cancer cells through the blood-brain barrier (Bos *et al.*, 2009) and genes involved in lung metastases have been recognised (Minn *et al.*, 2005). Although these studies point out the specific gene sets in organ specific metastases, there are numerous genes that are common for several types of tumours. These genes are considered to be importat for the initiation (e.g., VEGF, CSF-1, ID1, TWIST1, MET, FGFR, MMP-9, NEDD9) and progression (e.g., EREG, COX-2, MMP-1, CCL5, ANGPTL4) of metastases during the crucial steps prior to the organ-specific colonisation of cancer cells (Chiang and Massague, 2008). Many of these genes encode extracellular matrix (ECM) proteins or are related to EMT, and/or involved in the crosstalk between tumour cells and the surrounding microenvironment.

2.1.4 Regulation of breast cancer growth

Normal breast growth and development are regulated by the complex interaction of many hormones and growth factors. Some of these factors are secreted by the mammary epithelial cells themselves and may have autocrine functions, whereas others are produced by stromal cells and generate paracrine control on mammary epithelial cells. Thus, it is not surprising that the same growth regulatory hormone and growth factor systems are involved in the malignant growth of breast cancer cells. The interaction of growth factors, cytokines, and hormones with specific membrane receptors triggers a cascade of intracellular biochemical signals, resulting in the activation and repression of various subsets of genes. A simplified summary of some known growth stimulating signals that regulate breast cancer cell growth is illustrated in Figure 1; however, only some of them are described in detail in the following paragraphs.

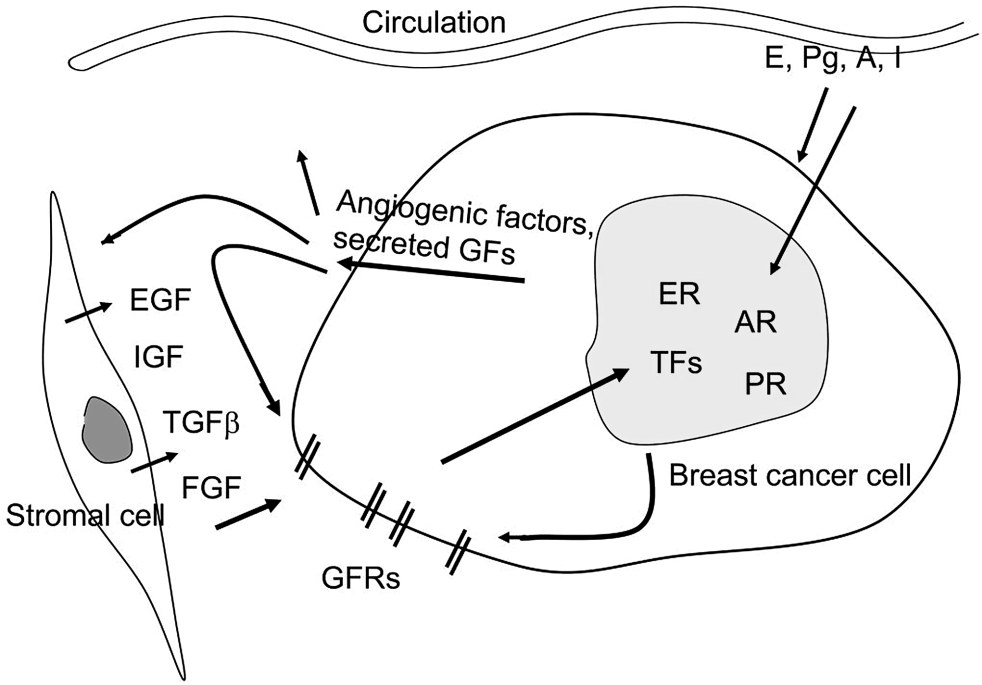

Figure 1. Mechanisms involved in the growth regulation of breast cancer cells. Components in the tumour microenvironment include stromal cells, immune cells (not shown here) and the vasculature. Growth factors (GFs) are secreted by both tumour and stromal cells, and they mediate their signals in an autocrine and paracrine fashion through their cognate cell membrane receptors (GFRs). Steroid and peptide hormones are provided via the circulation or produced locally within the tumour tissue, and they affect tumour cells interacting with their nuclear or cell membrane receptors, respectively. Growth factor -activated signalling pathways and ligand-bound nuclear receptors activate and/or interact with transcription factors (TFs), which in turn regulate the expression of numerous target genes, including oncogenes and tumour suppressor genes. A, androgens; AR, androgen receptor; E, oestrogens; EGF, epidermal growth factor; ER, oestrogen receptor; FGF, fibroblast growth factor; I, insulin; IGF, insulin like growth factor; Pg, progesterone; PR, progesterone receptor; TGFβ, transforming growth factor beta.

2.1.4.1 Hormone regulation of breast cancer growth

The steroid hormones known as oestrogens and progestagens are the main female sex hormones that regulate normal mammary gland development and function, and it is thought that breast cancer progression is largely influenced by them and their receptors. The role of the male sex hormones, androgens, is still partially unclear, but it has been reported that androgen levels do have an impact on the risk for breast cancer (rev. in Dimitrakakis and Bondy, 2009). In addition, the recent reports

regarding triple negative breast cancers have implicated a role for androgens in these tumours (Gucalp and Traina, 2010). The oestrogen receptors (ER), progesterone receptors (PR) and androgen receptor (AR) belong to the steroid hormone receptor super family, and they share a common overall structure: the well-conserved ligand- and DNA-binding domains, variable hinge and N-terminal regions (Mangelsdorf *et al.*, 1995). ER expression is found in 50-80% of malignant breast tumours, PR in 45% and AR in 70% of breast tumours (Kuenen-Boumeester *et al.*, 1992; Hall *et al.*, 1996). Importantly, the steroid hormones are thought to mediate their effects in the mammary gland largely by paracrine and autocrine target molecules such as growth factors and growth factor receptors (Dickson and Lippman, 1995).

2.1.4.1.1 Oestrogens

Oestrogens are well-characterised steroid compounds that function as primary female sex hormones that regulate the normal growth and differentiation of mammary tissue. More than a century ago, a connection between the ovaries, which is the main source of oestrogens in premenopausal women, and the growth of breast cancer was found (Beatson, 1986). Later, it became clear that oestrogens, in particular 17β-estradiol (E2), play an important role in the development and progression of breast cancer (Henderson *et al.*, 1988). E2 is secreted by the ovaries until menopause, but the production of oestrogens in the peripheral tissues is sustained or even increased after menopause. Moreover, E2 can also be produced in cells by conversion from androgen by an aromatase enzyme which is present in various cell types in the breast (Santner *et al.*, 1997). Oestrogens exert their cellular effects through the binding and activation of two distinct nuclear receptors, the well-characterised ERα originally cloned from MCF-7 breast cancer cells (Walter *et al.*, 1985) and the later identified ERβ (Kuiper *et al.*, 1996; Mosselman *et al.*, 1996). Classically, these receptors regulate gene transcription by binding to DNA at specific sites termed oestrogen response elements (EREs) (Klein-Hitpass *et al.*, 1988). However, around one-third of the known oestrogen regulated genes do not have EREs in their promoters and interact with ERs indirectly through intermediary transcription factors (O'Lone *et al.*, 2004). ER is generally expressed at low levels in normal breast epithelial cells (Ricketts *et al.*, 1991), but approximately 75% of malignant lesions express high levels of ER in the majority of cells (Pallis *et al.*, 1992; Zafrani *et al.*, 1994).

Oestrogens also have rapid, non-genomic mechanisms of action, which are proposed to be mediated by a small fraction of the traditional ER or perhaps by closely related splicing/translational variants of ER (Figtree *et al.*, 2003; Li *et al.*, 2003; Wang *et al.*, 2006), or by a G-protein coupled receptor named GPR30 (Thomas *et al.*, 2005). The rapid effects include activation of intracellular signaling pathways like MAPKs; induction of ion channel fluxes; and the generation of second messengers, such as cAMP (rev. in Lokuge *et al.*, 2010).

An intriguing discussion about the differential roles of ERα and ERβ has been underway since the discovery of ERβ. Contradictory reports were published regarding the role of ERβ in breast tumours. Some suggested an inhibitory and protective role (Mann *et al.*, 2001; Roger *et al.*, 2001), whereas others found correlation between ERβ and a high grade of breast tumours (Jarvinen *et al.*, 2000). In experimental studies, ERβ does, in fact, inhibit proliferation and promote differentiation in different cell types (Imamov *et al.*, 2004; Cheng *et al.*, 2005; Wada-Hiraike *et al.*, 2006). To date, there is strong evidence that ERβ acts as a protective factor against cancer, and by having often antagonistic features, the ratio of ERα and β is apparently one of the major determinants of the response to oestrogens in any given cell type (Heldring *et al.*, 2007; Warner and Gustafsson, 2010).

Many of the current treatment strategies for both early and advanced breast cancer are designed to block the mitogenic effects of oestrogens. Current antioestrogen treatments include selective oestrogen receptor modulators (SERMs) such as tamoxifen (Tam), raloxifene (Ral) and toremifene (Tor), selective oestrogen receptor downregulators (SERDs), and aromatase inhibitors (Hoffmann and Sommer, 2005). Due to their tissue-specific agonistic and antagonistic properties, SERMs are the optimal choice to block oestrogen action in the breast without increasing the risk for endometrial cancer and osteoporosis or cause other unwanted side effects that would result from complete inhibition of ER signalling throughout the body. In addition, SERMs have been studied in the context of the prevention of breast cancer in women at high risk for breast cancer, and thus far, tamoxifen has been proven to be successful in cancer intervention in premenopausal women (Fisher *et al.*, 1998). ER expression is generally considered a good prognostic marker and is tought to predict good response to therapies. However, approximately 50% of the patients with advanced breast cancer do not respond to Tam treatment, and moreover, many women who receive it acquire resistance to Tam when the disease progresses. The crosstalk between ER and growth factor pathways and the abnormal activation of the growth factor signalling are so far the best known mechanisms of developing endocrine resistance. For example, crosstalk between ER and the Her1/Her2 pathway has been shown to have an important role both in *de novo* and in acquired resistance to endocrine manipulation (Arpino *et al.*, 2009).

2.1.4.1.2 Progestagens

Progesterone is a steroid hormone that is responsible for maintaining and preparing the uterus for pregnancy, and it is secreted by the corpus luteum after ovulation. Progesterone is the major mitogen in the breast and it is involved in the mammary gland differentiation. Synthetic analogues of progesterone are called progestins, and they have been widely used as part of hormone replacement therapy (HRT) in postmenopausal women; however, a large study conducted some years ago, showed that exposure to progestins increases the risk of breast cancer in postmenopausal

women (Million women study 2003). This finding has led to re-evaluation of the therapeutic use of these compounds.

The PR is generally considered to antagonise oestrogen function (Kraus *et al.*, 1995), probably due to its ability to bind non-liganded ER and alter its function as a transcription factor (Ballare *et al.*, 2003). PR expression is upregulated by oestrogens (O'Lone *et al.*, 2004), and generally the presence of PR is considered a good prognostic factor and a predictive marker for the response to endocrine therapy in breast cancer. Loss of PR has been thought to be indicative of nonfunctional ER and the loss of hormone response (Osborne *et al.*, 1980). However, the function of PR is complicated. The PR is transcribed from a single gene via alternate usage of up to three independent translational start sites, resulting in PR-A, PR-B, and PR-C isoforms (Kastner *et al.*, 1990), of which PR-A and PR-B are expressed in breast tissue. PR-A and PR-B differ in their intracellular location and in their mitogen activated protein kinase (MAPK)-induced phosphorylation sites, transcriptional activities, turnover rates, protein complex formation and target gene specificity, leading to both proliferative and antiproliferative activity of PRs (Chakraborty *et al.*, 2010). PR, like the other steroid receptors, interacts with growth factor signalling. For example, the loss of PR is shown to be linked to high Her2 signaling (Hopp *et al.*, 2004). Recently, a membrane bound receptor, mPR, for progesterone has been characterised, further increasing the complexity of progesterone signalling (rev. in Dressing *et al.*, 2011).

2.1.4.1.3 Androgens

The role of androgens, mainly testosterones (Te) and dihydrotestosterones (DHT), in the normal physiology of women is not fully understood. During breast development, androgens generally have an inhibitory role that opposes oestrogen function (Labrie, 2006). Regarding breast cancer, it remains controversial whether androgen levels reduce or increase the risk for the disease. Several epidemiological studies show an association with high androgen levels and an increased risk of breast cancer (Key *et al.*, 2002; Kaaks *et al.*, 2005; Tamimi *et al.*, 2006; Micheli *et al.*, 2007), but there are also reports that show that increased androgens levels may, in fact, be protective against breast cancer (Dimitrakakis *et al.*, 2004; Ogawa *et al.*, 2008). In addition, some studies have not found any correlation between androgen levels and breast cancer (Adly *et al.*, 2006; Cox *et al.*, 2006). In male breast cancer, androgens have a protective function; a relative excess in oestrogen or a lack of androgens increase the risk for breast cancer (rev. in Gomez-Raposo *et al.*, 2010). There is evidence that polymorphisms in the AR gene could be associated with an increased or reduced risk for breast cancer, but no consensus on this issue has yet been achieved (Dimitrakakis and Bondy, 2009). The contradictory observations in the field have been proposed to be at least partly due to the impact of low versus high oestrogen levels that could affect the agonistic/antagonistic action of androgens and their metabolites and by the fact that circulating steroid levels do not always reflect the actual concentrations inside the cells and tissues (Nicolas Diaz-Chico *et al.*, 2007).

AR is expressed in normal mammary epithelium and in the majority of breast cancer specimens and cell lines. There is a clear association between the expression of AR, ER and PR (Kuenen-Boumeester *et al.*, 1992); however, AR is also expressed independently of other steroid receptors in a portion of breast cancers, and it has a significant association with important prognostic factors (Isola, 1993; Agoff *et al.*, 2003). *In vitro*, androgens have been shown to have both inhibitory and stimulatory effects depending on the cell line, their concentration and the ER status of the cell line (Birrell *et al.*, 1995; Zhou *et al.*, 2001; Somboonporn *et al.*, 2004). The stimulatory effects on breast cancer cells have been suggested to result, at least partly, from aromatase enzyme activity, that is, from the ability to convert androgens to oestrogens (Santen *et al.*, 1998). However, there is emerging evidence that androgens have important direct functions via AR in producing tumours with specific characteristics, especially in ER-negative breast cancers (Farmer *et al.*, 2005; Nicolas Diaz-Chico *et al.*, 2007). Interestingly, a microarray profile of a set of breast cancers identified a subtype of ER/PR-negative tumours that express AR and have a gene expression profile close to that of ER-positive tumours. These tumours were named as ER(-) class A tumours (Doane *et al.*, 2006). Interestingly, in MDA-MB453 cell line, which has a similar receptor status and gene expression profile as these tumours, the cell growth and survival were highly responsive to androgens in an AR-dependent manner, suggesting AR blockage to be potential therapeutic strategy for this subset of tumours. The first phase II clinical trial in which the efficacy of the antiandrogen, bicalutamide, is being studied in patients with ER-/PR-/AR+ advanced breast cancers is currently underway (Gucalp and Traina, 2010). An interesting detail from the perspective of this thesis is that AR expression has been associated with FGF-8 expression in breast cancer (Tanaka *et al.*, 2002). Furthermore, in the S115 model used in this study, androgens act as malignant transformation and growth stimulating factors. Altogether, it is evident that AR has relevance in breast cancer biology, and it may serve as an important target at least in TNBCs that express AR.

2.1.4.2 Growth factor regulation of breast cancer growth

Self-sufficiency in growth signals is one of the six hallmarks of cancer that were introduced by Douglas Hanahan and Robert Weinberg in their famous review at 2000 (Hanahan and Weinberg, 2000). Through the enhanced secretion of soluble mitogenic growth factors (GFs) that act in an autocrine/paracrine fashion and by altering GF receptor functions, tumour cells can escape from their dependence on exogenous growth stimulation and regulation. In breast tissue, the interaction between steroid receptors and growth factor signalling is essential to the regulation of normal development and function. However, altered growth factor signalling is thought to play an important role in the endocrine insensitivity and acquired resistance to hormone therapy in breast cancer (Nicholson and Gee, 2000). Of the several known growth factor families, the EGF, IGF and TGFβ signalling pathways have a well-documented role in the development of breast cancer, and they will be briefly described in the following paragraphs. In addition to these pathways, an increasing amount of data shows that FGF signalling is one of the major players in the growth of

breast cancer cells. The FGF/FGFR protein families will be discussed in detail in Chapter 2.2. As growth factor receptors and the associated signal transduction molecules serve as potential drug targets, these pathways have great potential and provide hope for better treatment strategies, especially for the endocrine-resistant metastatic breast cancers, in the future.

2.1.4.2.1 Epidermal growth factor family

The epidermal growth factor family (EGF) is important for mammary gland morphogenesis and in ductal and lobuloalveolar growth. The EGF family consists of several peptide growth factors, of which at least EGF, transforming growth factor-alpha (TGF-α) and amphiregulin have been implicated to be involved in the malignant growth of breast tissue (rev. in Booth and Smith, 2007; McBryan *et al.*, 2008). EGF ligands serve as agonists to the human EGF receptor family (EGFR/ErbB/HER) which comprises four closely related tyrosine kinase receptors (EGFR1-4/ErbB1-4/Her1-4). The nomenclature of these receptors varies, but EGFR1 is most commonly referred to as EGFR or ErbB1, and EGFR2 as Her2 or ErbB2. The EGF family ligands exhibit a complex pattern of interactions with the four ErbB receptors, leading to divergent biological outcomes (Wilson *et al.*, 2009).

Generally, ligand binding to ErbB1 or ErbB4 leads to dimerisation of the receptor and activation of the intracellular tyrosine kinase (TK), which then transduces the signal further to downstream Ras-Raf-MAPK, phosphatidyl-inositol-3-kinase (PI3K)/Akt, signal transducer and activator of transcription (STAT) and Src pathways (Lurje and Lenz, 2009). The activated pathway depends on the ligand and the receptor dimer complex, as well as on the cellular context. Aberrant ErbB activation has long been considered to be involved in several phenomena in malignant growth, such as cellular proliferation, apoptosis, angiogenesis and metastasis (Hemming *et al.*, 1992; Salomon *et al.*, 1995).

Increased ErbB1 and Her2 expression have been linked to decreased endocrine sensitivity in breast cancer. Her2 amplification occurs in 20-30% of invasive ductal carcinomas, and it has been shown to be involved in increasing the proliferation and survival of the primary tumour as well as in breast cancer metastasis. Interestingly, Her2 was shown to be one of the few cases in which a perfect association between the gene amplification and over-expression exists (Press *et al.*, 2002), although over-expression in some breast cancers may also be due to the deregulation of enhancer elements near the Her2 promoter (Bosher *et al.*, 1995; Bosher *et al.*, 1996). Nevertheless, an association between the amplification of Her2 leading to its over-expression and the survival of breast cancer patients (Slamon *et al.*, 1987) led to the development of targeted therapeutics, such as the monoclonal antibody trastuzumab (Herceptin, Genentech Inc.), which are currently in use for the treatment of metastatic breast cancer. However, a large percentage of Her2-positive breast cancers are or

become resistant to Her2-targeted therapies, which is possibly caused by adaptive and genetic changes in cancer cells (Wang and Greene, 2008).

2.1.4.2.2 Insulin-like growth factor family

The insulin-like growth factor (IGF) family consists of two factors: IGF-I and IGF-II. They are small 7-kD polypeptides with a high degree of homology to insulin (Zhang and Yee, 2000). Most of the circulating IGF-I is produced by the liver, but IGF-I is also produced in other tissues where it acts in a paracrine and/or autocrine fashion. The normal physiological function of IGFs is associated with the regulation of cell proliferation and apoptosis in relation to nutrient availability, but regulatory roles related to energy metabolism, body size, longevity and various organ-specific functions are also evident. IGFs bind to IGF binding proteins (IGFBPs), IGF-I receptors (IGF-IR1 and -2) and insulin receptors (IR-A and IR-B), which mediate the signal further to intracellular PI3K and MAPK cascades (Zhang and Yee, 2000). Elevated plasma IGF-I levels are associated with an increased incidence of many cancers, including breast cancer (Pollak *et al.*, 2004). Both IGF-R1 and IR are overexpressed in breast cancer (Papa *et al.*, 1990; Papa *et al.*, 1993), but controversial reports have been published regarding the clinical significance of the over-expression (Lann and LeRoith, 2008).

IGF signalling has been well documented to have crucial role in the normal development of the mammary gland. Briefly, growth hormone (GH)-induced IGF acts synergistically with oestrogens to induce terminal end bud (TEB) formation and ductal morphogenesis in mice (Kleinberg, 1997; Ruan and Kleinberg, 1999). Cross-talk between oestrogen and IGF-signalling may also be critical for the oestrogen-mediated growth of breast cancer (Lee *et al.*, 1999; Yee and Lee, 2000). In breast cancer experimental models, IGF-I induces cell proliferation (Pollak *et al.*, 1988) and increases the frequency of mammary tumours (Hadsell *et al.*, 2000), and its downregulation delay chemically or genetically induced mammary tumourigenesis (Wu *et al.*, 2003). Accordingly, the overexpression of IGF-R1 results in the development of mammary adenocarcinomas (Carboni *et al.*, 2005). Altogether, IGF signalling has a clear tumour growth-promoting role; thus, the IGF system could be utilised as a target for breast cancer therapy (Sachdev and Yee, 2007).

2.1.4.2.3 Transforming growth factor beta family

The transforming growth factor beta (TGFβ) family consists of numerous ligands such as TGFβ1-3, activins, inhibins and bone morphogenetic proteins. The most famous member of this family, TGFβ, is no doubt one of the most extensively studied growth factors in mammary gland development and breast cancer. In early studies, TGFβ was shown to act as a tumour suppressor in the breast (Silberstein and Daniel, 1987), but some years later, it was shown that the inhibitory role of TGFβ can be overruled by

breast cancer cells (Basolo *et al.*, 1994). Today, it is generally accepted that TGFβ has a dual role in the tumour growth: TGFβ has a tumour suppressive role in the early growth phase and a tumour promoting role in the later stages of cancer progression (Barcellos-Hoff and Akhurst, 2009). Interestingly, members of the TGFβ family of cytokines are the main and the best characterised inducers of EMT during the course of embryonic development, wound healing, in fibrotic diseases and in cancer pathogenesis (Massague, 2008). Recent data also implicate a role for TGFβ in regulating breast cancer stem cell phenotypes (Mani *et al.*, 2008; Morel *et al.*, 2008).

TGFβ is secreted as an inactive latent complex consisting of a TGFβ dimer and prosegments that must be removed to release the highly stable and active TGFβ dimer (Derynck *et al.*, 2001). Several different proteases can activate TGFβ, including plasmin (Lyons *et al.*, 1990) and matrix metalloproteases (MMP) 2 and 9 (Yu and Stamenkovic, 2000). TGFβ signals through a heteromeric cell-surface complex of two types of transmembrane serine/threonine kinases named type I (TβRI) and type II (TβRII) TGFβ receptors. The binding of TGFβ results in the phosphorylation of TβRI or II, which then leads to the signalling of downstream target proteins, of which the best known is the Smad pathway. Smads regulate several transcription factors, leading to activation or inhibition of the transcription of TGFβ target genes (Derynck *et al.*, 1998; Massague and Wotton, 2000; Massague, 2000).

2.1.4.2.4 Angiogenic factors in breast cancer

There are numerous factors that act as mediators of pro- and antiangiogenic signals. The angiogenic factors can belong to different growth factor or ECM protein families, and they are usually secreted by different cell types or at different stages of normal development or tumour growth. They are commonly classified as angiogenic factors based on either their ability to induce or promote angiogenesis in experimental models or on their documented role in normal physiology. Of the defined angiogenic factors, at least the VEGF family members, FGFs, PDGFR, PlGF, TGFβ, thymidine phosphorylase (Relf *et al.*, 1997), and the angiopoietins Ang1 and Ang2 (Hayes *et al.*, 2000; Sfiligoi *et al.*, 2003) have been reported to be expressed by breast cancer cells or tumours. In addition, proteins such as ephrins, endothelins, integrins, cadherins and Notch family members have been implicated in the regulation of tumour angiogenesis (Gordon *et al.*, 2010). Proteins classified as endogenous angiogenesis inhibitors include e.g., thrombospondins, angiostatins and endostatins (Ribatti, 2009). Here, only one example of a pro- and one antiangiogenic factor relevant to this thesis study are presented in detail, namely vascular endothelial growth factor (VEGF) and thrombospondin-1 (TSP-1), respectively. The balance of TSP-1 and VEGF is largely influenced by the tumour microenvironment where the communication between tumour cells and the cells in the tumour-associated stroma takes place (Kang and Watnick, 2008).

2.1.4.2.4.1 VEGF

Together with placental growth factors (PlGFs), VEGF-A,-B,-C and -D comprise the VEGF family of secreted glycoproteins, which are important in angiogenesis and lymphangiogenesis. VEGF was originally discovered in 1970 as an unnamed angiogenic factor (Folkman *et al.*, 1971), and approximately ten years later, it was identified as a factor that increases vascular permeability in the vessels lining the peritoneal cavities of mammals with ascites tumours (Senger *et al.*, 1983). To date, VEGF has a well-established role in the tumour angiogenesis, and VEGF/VEGFR pathways are known to be essential for vascular homeostasis (Lee *et al.*, 2007). The expression of VEGF is induced by hypoxia via HIF, the essential regulator of hypoxia-sensitive genes (Maxwell, 2005). VEGF expression is also regulated by growth factor signalling, and TGFs, IGFs, FGFs and PDGF have been shown to upregulate VEGF expression (Robinson and Stringer, 2001). In addition, VEGF expression is under hormonal regulation by sex steroids, and oestrogens, androgens and progestins are reported to induce or maintain its expression at least in human endometrial tumours and endometrial cancer cell lines, in breast and prostate cancer cells and in prostate tumours (Ruohola *et al.*, 1999; Mueller *et al.*, 2000; Mueller *et al.*, 2003; Garvin *et al.*, 2005). However, there are conflicting data about the relationship between ER and VEGF: high ERα levels have been shown to down-regulate VEGF (Ali *et al.*, 2000), and ERα status and VEGF have been shown to be inversely correlated (Fuckar *et al.*, 2006). Other studies have shown high VEGF levels, particularly in ER-positive breast cancers (Heer *et al.*, 2001). Nevertheless, it is clear that the over-expression of VEGF is associated with tumour progression and poor prognosis in breast cancer (Foekens *et al.*, 2001; Nakopoulou *et al.*, 2002; Banerjee *et al.*, 2007).

To mediate its signal, VEGFs bind to the three VEGF receptors, VEGFR1 (Flt-1), VEGFR-2 (KDR/Flk-2) and VEGFR-3 (Flt-4), which have distinct roles in the angiogenic and lymphangiogenic processes. In addition, VEGFs bind to Neuropilin-1 and -2, which also have roles in tumour progression (Bielenberg *et al.*, 2006). VEGF binding to VEGFRs induces downstream signalling networks such as the PI3K, PLCγ, and MAPK pathways, but the details of VEGFR signal transduction and the significance of the different pathways in certain responses are still largely unclear. It is known that VEGFR-1 and -2 have different functions. VEGFR-1 is likely involved in angioblast differentiation and maintenance, whereas VEGFR-2 is a regulator of endothelial cell proliferation, migration and survival; thus, VEGFR-2 has become an important target for drug development (Carpini *et al.*, 2010). VEGFs affect endothelial cell proliferation, survival and migration, and moreover, they are involved in recruiting endothelial progenitor cells to the sites of neovascularisation.

2.1.4.2.4.2 TSP-1

Thrombospondin 1 (TSP-1) is a multifunctional ECM protein. It was originally isolated as a component of platelet alpha-granules (Baenziger *et al.*, 1972) and was

later recognised as the first endogenous antiangiogenic protein (Good *et al.*, 1990). Together with TSP-2, it forms the group A thrombospondins within the thrombospondin protein family. TSP-1 is a 450-kd homotrimeric protein expressed ubiquitously by both normal and tumour cells. TSP-1 consists of a number of functional domains (presented in Figure 2) that allow it to interact with cells and other proteins (Sid *et al.*, 2004). The antiangiogenic activity of TSP-1 has been mapped to the pro-collagen domain and three three type I repeats (TSRs; rev. in Lawler and Detmar, 2004). The mechanism of the antiangiogenic action of TSP-1 involves binding to CD36, leading to the inhibition of endothelial cell proliferation and migration, along with the induction of apoptosis in these cells (Armstrong and Bornstein, 2003). It has also been shown to play a role in antitumour immunity by recruiting tumour-associated macrophages and by enhancing tumour cell death via the release of reactive oxygen species (Martin-Manso *et al.*, 2008). These findings suggest that the TSP-1 antitumour activity is not only due to its antiangiogenic activity. The downregulation of TSP-1 has generally been considered an important element in the switch to a more malignant and angiogenic phenotype (Sid *et al.*, 2004).

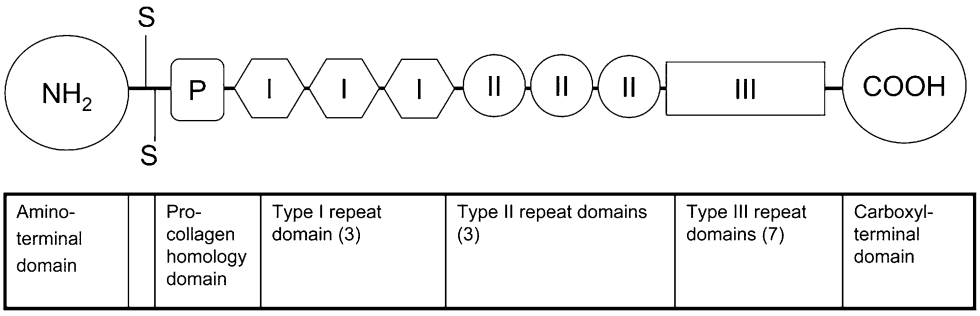

Figure 2. Schematic presentation of the multimodular structure of a single subunit of TSP-1. Modified from Sid *et al*. 2004.

By mediating cell-matrix interactions, TSP-1 is involved in several biological processes, including cell adhesion, migration, differentiation, proliferation and apoptosis. TSP-1 expression is regulated by several known tumour suppressor genes and oncogenes such as p53, PTEN, Ras and Myc (rev. in Ren *et al.*, 2006). It is widely accepted that TSP-1 inhibits angiogenesis and the growth of primary tumours, but its role in tumour metastasis remains controversial. There have been several studies that show TSP-1 to promote the invasion and metastasis of breast cancer cell lines (Albo *et al.*, 1997) and recently in a transgenic Pyt mouse breast cancer model (Yee *et al.*, 2009). It is possible that because of its multidomain structure, TSP-1 can engage different receptors that affect different signalling pathways depending on the spatial and temporal context. Its interaction with several cytokines, growth factors and

proteinases such as TGFβ, FGFs, MMP2, VEGF and PDGFs may also be important for the outcome of TSP-1 effects (Lawler, 2000).

In human breast cancer, TSP-1 is highly expressed by stromal fibroblasts, macrophages and endothelial cells (Brown *et al.*, 1999), but its expression is often reduced or lost in tumour cells (Watnick *et al.*, 2003; Naumov *et al.*, 2006). However, plasma TSP-1 levels are higher in advanced breast cancer patients than in early stage breast cancer patients (Byrne *et al.*, 2007). Recent reports have shown that TSP-1 in breast tissue is under hormonal control by sex steroids and that it may have direct proliferative effects on breast cancer cells (Hyder *et al.*, 2009a; Hyder *et al.*, 2009b). Altogether, the role of TSP-1 in tumour growth seems to be complex and dependent on the cellular context, cell type and the hormonal milieu.

2.2 FIBROBLAST GROWTH FACTORS

Fibroblast growth factors (FGFs) and their receptors (FGFRs) play an important role in a variety of processes during embryonic development and tissue homeostasis. They are extensively studied mitogenic factors in most cell types and their importance has been described in several recent in-depth reviews of processes like wound healing and tissue repair (Beenken and Mohammadi, 2009), angiogenesis (Presta *et al.*, 2005), cholesterol metabolism and serum phosphate regulation (Kharitonenkov, 2009) and in cancer (Turner and Grose, 2010). In the following chapters, an overall summary of the FGF and FGFR protein families and their signalling pathways is given, with special interest in the recent advances in FGF research in mammary biology and breast cancer. However, all the FGF-related processes mentioned above are not discussed here.

2.2.1 FGF protein family

The first members of the FGF protein family were found after studying the mitogenic action of bovine pituitary and brain tissue, leading to the isolation of basic FGF (bFGF) and acidic FGF (aFGF), which were later renamed as FGF-2 and FGF-1, respectively (rev. in Mohammadi *et al.*, 2005; Tanaka, 2005). To date, the FGF family in vertebrates comprises 22 members, 18 of which are ligands for FGF receptors (FGFRs). Four family members (FGF11-14) are FGF homologous factors that do not function as FGF ligands. FGFs are secreted 17- to 34-kD glycoproteins with a highly conserved gene structure and amino acid sequence. Most of the FGF genes are scattered throughout the genome, but there are also several FGF gene clusters indicating that the FGF family was generated by gene and chromosomal duplication and translocation during evolution (Ornitz and Itoh, 2001). Most FGFs (3-8, 10, 15, 17-19, 21-23) contain an amino (N)-terminal signal peptide and are readily secreted from cells. FGFs -9, -16 and -20 in turn have an uncleavable N-terminal hydrophobic sequence that is required for their secretion (Miyamoto *et al.*, 1993; Miyake *et al.*,

1998a; Ohmachi *et al.*, 2000). FGF-1 and FGF-2 lack the signal peptide and are not secreted by the endoplasmic reticulum-Golgi pathway, but they can be released either from damaged cells or by an exocytotic mechanism (Mignatti *et al.*, 1992). After secretion FGFs are sequestered to the ECM and on cell surfaces by heparin sulphate protein glycans (HSPGs). To signal, FGFs must be released from the ECM by heparinases, proteases or specific FGF-binding proteins. Liberated FGFs can then bind to cell surface HSPGs and FGFRs. Although FGFs are thought to act mainly as secreted factors, there is evidence of nuclear localisation and function for some FGFs (Chlebova *et al.*, 2009).

The human FGFs are divided to several subfamilies, namely the FGF-1, FGF-4, FGF-7, FGF-8, FGF-9 and FGF-19 subfamilies. Members of each subfamily share sequence similarity and biochemical as well as developmental properties (Ornitz and Itoh, 2001). They tend to have a similar pattern of expression, although each FGF also has unique sites of expression. Some FGFs are expressed exclusively during embryonic development, whereas others are expressed both in embryonic and adult tissues. Most of the FGF ligands function in a classic autocrine or paracrine fashion. However, FGF-19, -21 and -22 act as hormones; they bind poorly to the HSPGs and can diffuse from the producing cells into the circulation. FGFs have a homologous core region of 120-130 amino acids that are ordered into 12 antiparallel β-strands. Sequence variation in the N- and C-terminal tails determines the different functions of the ligands (Mohammadi *et al.*, 2005). Moreover, FGF-8 and FGF-17 transcripts are subjected alternative splicing, yielding to several isoforms that differ in their N-termini (Crossley and Martin, 1995; Xu *et al.*, 1999). These FGFs and their isoforms are described in more detail in Chapter 2.2.9.1.

2.2.2 FGF receptors

FGFs exert their actions through four highly conserved tyrosine kinase receptors FGFR1, FGFR2, FGFR3 and FGFR4. There is also a fifth related receptor, FGFR5 (also known as FGFRL1), which can bind FGFs but it has no tyrosine kinase domain, thus it might negatively regulate FGF signalling (Wiedemann and Trueb, 2000; Sleeman *et al.*, 2001; Steinberg *et al.*, 2010). The binding of FGFs to FGFRs is dependent on the presence of HSPGs on the cell membrane. HSPGs serve as low affinity receptors for FGFs that facilitate the binding to FGFRs and stabilise the resulting FGF-FGFR complexes (Schlessinger *et al.*, 2000). Furthermore, HSPGs, such as Syndecans, can modulate FGFR signalling in both inhibitory and stimulatory fashion. Importantly, several FGFR isoforms are expressed in a tissue-specific manner, which enables directional and reciprocal signalling across epithelial-mesenchymal boundaries. Most FGFs bind several FGFRs, resulting in a high degree of functional redundancy within the system. The structure and function of the FGFRs and their isoforms are described in detail in following chapters.

2.2.2.1 Structure and expression of FGFRs

FGFR1-4 consist of three extracellular immunoglobulin domains (IgI-IgIII), a single-pass transmembrane helix and a cytoplasmic tyrosine kinase domain (represented in Figure 3). Between the IgI and IgII domains is an acidic region termed the acid box that is important in the autoinhibition of the receptor (Wang *et al.*, 1995). The IgII domain contains a conserved positively charged region that serves as a binding site for heparin (Schlessinger *et al.*, 2000). The IgII-IgIII fragment, in turn, is necessary and sufficient for ligand binding and specificity (Mohammadi *et al.*, 2005). There are several FGFR isoforms which result from alternative splicing of the primary FGFR mRNA transcripts. In FGFR1-3, the IgI domain and/or the acid box can be removed by exon skipping. FGFRs lacking these domains are capable of binding the ligands and transmitting responses, which suggests that the IgI domain and the acid box are dispensable for the receptor function (Mohammadi *et al.*, 2005). Alternative splicing of the exons 8 and 9, which encode the second half of the IgIII domain of FGFR1-3 yields the IgIIIb or IgIIIc isoforms, which have distinct ligand binding specificities. Distinct from the other FGFRs, FGFR4 is expressed as a single IgIII isoform that is paralogous to IgIIIc.

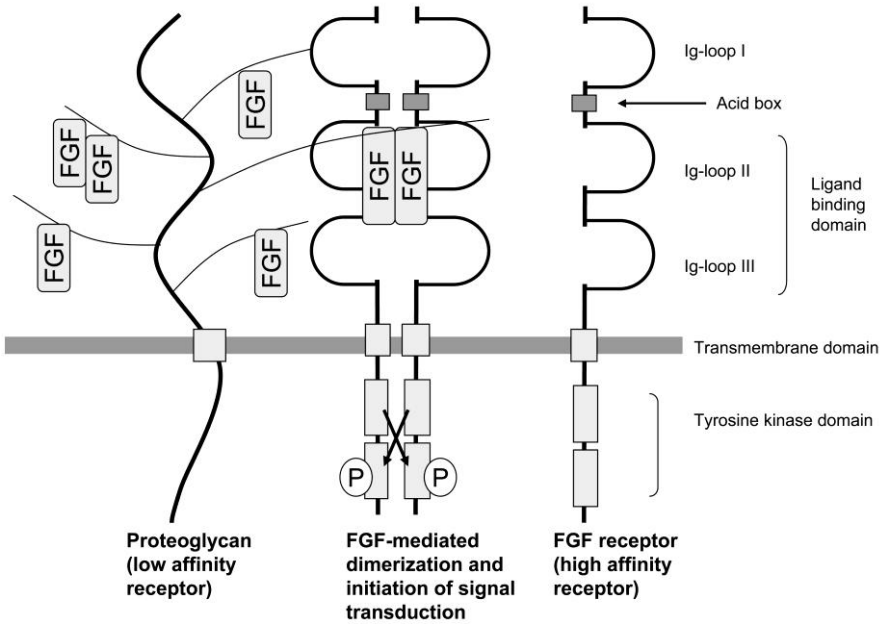

Figure 3. Structure of the FGFRs. Modified from Dickson *et al*. 2000.

An important feature of FGFR function is that the alternative splicing events occur in a tissue specific fashion, FGFR1-3IgIIIb forms are being expressed by epithelial cells and IgIIIc by mesenchymal cell lineages (Orr-Urtreger *et al.*, 1993). As

demonstrated by the first time for FGF7, many of the FGF ligands are also specifically secreted either by epithelial or stromal cells (Rubin *et al.*, 1989) or, like FGF-4, in a strictly regulated manner at a specific developmental stage (Niswander and Martin, 1992). Thus the FGF-FGFR signalling system provides a directional and spatial paracrine communication route between the epithelial and mesencymal compartments that is crucial for organogenesis and pattern formation during embryogenesis (Martin, 1998; Kato and Sekine, 1999) as well as for the maintenance of normal tissue homeostasis in adult organisms. The directionality of FGF-FGFR signalling based on the tissue-specific expression of FGFR isoforms and ligands is demonstrated in Figure 4.

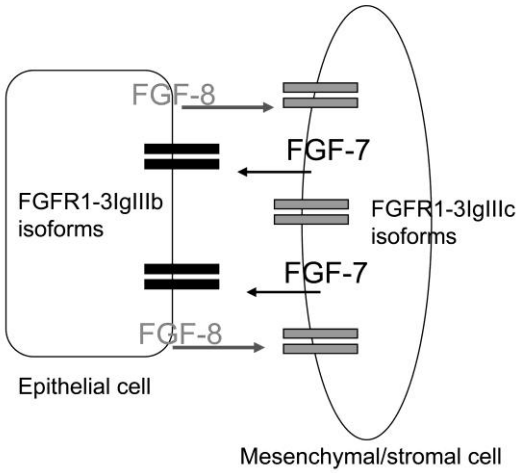

Figure 4. Directional FGF-mediated paracrine communication between the epithelial and mesenchymal compartments.

Based on their restricted expression pattern, FGFR1-3IgIIIb and IgIIIc isoforms are often considered to reflect the cell type in which they are being expressed, and they are predictive of the type of responses they transduce. In the case of cancer cells, the IgIIIb form is considered a marker for an epithelial phenotype and IgIIIc for a more aggressive and undifferentiated phenotype. Cells expressing IgIIIc may have undergone EMT. However, splice variants of FGFRs other than those differing in their IgIII domains may also differ markedly in terms of their function. Studies with human breast cancer cell line, SUM-52PE, have highlighted the importance of the intracellular C-terminus of the FGFRs (Moffa *et al.*, 2004; Moffa and Ethier, 2007). In this particular cell line, FGFR2IgIIIb is the dominant isoform. However, the cells were shown to express nine splice variants of the FGFR2IgIIIb, all of which have a similar ectodomain but differences in their intracellular domains. Strikingly, the transforming potential and the effects on the signal strength and duration was largely dependent on the alternatively spliced C-terminus of the receptor, and expression of a cancer-cell specific FGFR2-C3 form was shown to result in constitutively active receptor signalling (Moffa *et al.*, 2004; Moffa and Ethier, 2007).

2.2.3 FGFR activated signalling pathways

Fibroblast growth factor receptors signal as dimers and a functional FGF-FGFR consists actually of two FGF-FGFR-HSPG complexes, as was presented in Figure 2. Dimerisation of the receptors is a ligand-dependent event in which the cytoplasmic domains of the receptors are brought into close proximity to one another, enabling *trans* autophosphorylation to occur (Yarden and Ullrich, 1988). Phosphorylation of the tyrosine residues on the receptors' C-termini produces docking sites for adaptor proteins, which then transduce the signal further, eventually leading to a plethora of biological responses.

The main intracellular signal transduction pathways activated by FGFRs are well documented and presented in Figure 5 (Dailey *et al.*, 2005; Acevedo *et al.*, 2009). The activation of FGFR leads to the strong activation of the MAPK cascade, activation of phospholipase C gamma (PLCγ), and the PI3K pathways. In addition STAT and Src family members are activated by FGFRs. Importantly, several adaptor proteins are required to mediate the signal from the activated FGFR to these different signalling cascades. Fibroblast growth factor receptor substrate-2α (FRS2α) is a key adaptor protein in the FGF signalling axis that associates with the juxtamembrane region of inactivated FGFRs in a constitutive, phosphotyrosine-independent manner (Ong *et al.*, 2000). FRS2α contains myristyl anchors and phosphotyrosine binding (PTB) domains in its N-terminus and large regions with multiple phosphorylation sites at its C-terminus. FRS2α also has four binding sites for the adaptor protein Grb2 (growth factor receptor-bound 2) and two binding sites for the protein tyrosine phosphatase Shp2, which are important for its function. Upon phosphorylation resulting from FGFR activation, FRS2α forms a complex with Grb2 both directly by its own Grb2 binding domains and indirectly via Shp2 (Hadari *et al.*, 2001). Through Grb2, the FRS2 signalling complex recruits the guanine nucleotide exchange factor SOS (son of sevenless), which activates Ras and the downstream RAF and MAPK pathways including the ERK, p38 and JNK1/2 pathways (Eswarakumar *et al.*, 2005).

In addition, FRS2α is involved in the activation of the PI3K pathway through another docking protein, Grp2-associated binding protein Gab1 (Ong *et al.*, 2001; Lamothe *et al.*, 2004). Thus, FRS2α plays a key role in FGF signal transduction by coordinating the assembly of several docking proteins. The central role of FRS2α was strikingly demonstrated in FRS2α-/- mice, in which the targeted disruption of the FRS2α gene caused a severe impairment of mouse development resulting in embryonal lethality at E7.0-7.5. The embryonal fibroblasts of these embryos showed severe defects in the signalling events described above (Hadari *et al.*, 2001). However, disrupting FRS2α did not change the activation of PLCγ. Indeed, PLCγ binds directly to a specific binding site in the C-terminal tail of activated FGFR through its Src homology (SH2) domain and becomes phosphorylated independently of FRS2α. PLCγ activation leads to the stimulation of phosphatidylinositol (PI) hydrolysis and the generation of two second messengers, PI-2,3,4-inositol (PIP3) and diacylglyserol

(DAG), eventually resulting in the activation of protein kinase C (PKC) and the phosphorylation of several target proteins. In addition, the non-receptor tyrosine kinase, Src, is activated upon FGF stimulation *in vitro* (Landgren *et al.*, 1995) and it is shown to contribute to cell migration and the dynamics of FGFR1 signalling (LaVallee *et al.*, 1998; Sandilands *et al.*, 2007). Furthermore, the FGFR-induced activation of STAT1 and STAT3 is thought to play a role in cell proliferation and survival (Hart *et al.*, 2000). A schematic summary of the best known FGFR-activated signalling pathways is presented in Figure 5.

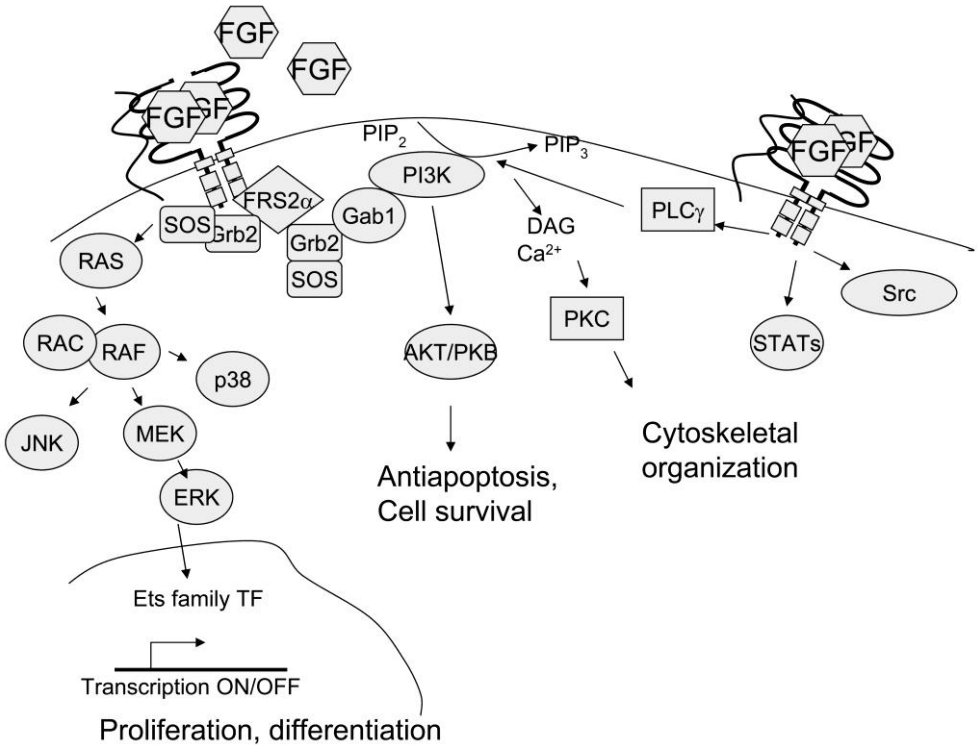

Figure 5. Simplified representation of the FGF signalling network. Modified from Acevedo *et al*. 2009 and Dailey *et al*. 2005.

2.2.4 Cellular responses to FGF-FGFR signalling

The diversity of biological responses this growth factor system produces in different cell types and tissues is an intriguing topic and a source of numerous open questions in the FGF-FGFR research field. The activation of the different signalling pathways described above leads to broad spectrum of responses, ranging from cellular

proliferation and regulation of cell death, to the modulation of cytoskeletal organisation and inflammatory responses (Dailey *et al.*, 2005). Of the activated MAPK pathways, the ERK1/2 kinases are generally considered to be responsible for the mitogenic responses, while the p38 and JNK pathways are associated with inflammatory or stress-responses (Johnson and Lapadat, 2002). The PI3K pathway is classified as an anti-apoptotic pathway that promoted cell survival (Vivanco and Sawyers, 2002). However, several studies have shown that no simple correlation can be made between the activation of a particular pathway and the biological response to FGF. Therefore, even though FGFs trigger similar signalling cascades in all cell types, the response depends largely on the cellular context. An example of the paradoxical responses resulting from the activation of a single pathway comes from studies with FGF-induced ERK1/2 activation, which has been shown to promote proliferation in oligodendrocytes and endothelial cells (Tanaka *et al.*, 1999; Baron *et al.*, 2000) but cause cell cycle arrest in chondrocytes (Raucci *et al.*, 2004). Similarly, FGFs can either promote or inhibit the differentiation of chondrocytes and osteoblasts, respectively (Mansukhani *et al.*, 2000; Dailey *et al.*, 2003). A characteristic feature of FGF signalling is that the response to FGF stimulation is not only cell type-specific but is also dependent on the cellular differentiation stage (Jacob *et al.*, 2006), and the magnitude and duration of FGF stimulation (Garcia-Maya *et al.*, 2006) even in the same cell type. Thus, "context-dependent signalling" is a key concept in FGF signalling (Turner and Grose, 2010).

2.2.4.1 Differential FGFR signalling in breast cancer cells

Despite the structural and functional similarity and the shared signalling pathways, the different FGFRs do activate signalling cascades distinctly. Important data regarding this issue in context of breast cancer have been produced by Dr. Jeffrey Rosen's research group. They generated mouse models in which ligand-dependent drug-inducible FGFR1 and FGFR2 (iFGFR1 and iFGFR2) were expressed under the mouse mammary tumour virus (MMTV) promoter, resulting in mice in which FGFR1 or FGFR2 can be activated in mammary tissue independently of the activation of other FGFRs and of endogenous ligands (Welm *et al.*, 2002). Activation of iFGFR1 in these mice rapidly led to progressively invasive lesions, ECM remodelling and vascular branching in the stroma adjacent to these lesions. Interestingly, when primary mammary epithelial cells (MECs) from these MMTV-iFGFR1 and MMTV-iFGFR2 mice were cultured in 3D-cultures, the activation of iFGFR1 or iFGFR2 could both induce the disruption of cell polarity and promote cell proliferation; however, they also exhibited marked differences in their regulation of apoptosis and EMT. The activation of iFGFR1 promoted EMT and decreased apoptosis, whereas the activation of iFGFR2 induced apoptosis and failed to promote EMT (Xian *et al.*, 2007). Accordingly, iFGFR1 activation in immortalised mouse mammary epithelial HC11 cells in 3D-culture rapidly disrupted cell polarity, induced proliferation and promoted cell survival (Xian *et al.*, 2005). Unfortunately, a detailed characterisation of the MMTV-iFGFR2 mice was not published, which leaves open the question of whether

these mice also developed hyperplastic lesions or not. Nevertheless, *in vitro* studies of iFGFR1 and iFGFR2 cell lines have shown that the mechanisms behind the more aggressive phenotype of iFGFR1-activated cells included more sustained ERK activation and secretion of MMPs (Xian *et al.*, 2005; Xian *et al.*, 2007). Moreover, the growth and survival of these cells was dependent on the activity of p90 ribosomal S6 kinase (RSK), which acts downstream of ERK (Xian *et al.*, 2007; Xian *et al.*, 2009). The activation of ERK in response to iFGFR2 stimulation was, in turn, attenuated through the rapid downregulation of the receptor, which was dependent on the ubiquitin ligase Cbl activity (Xian *et al.*, 2007). In a very recent study from the Rosens laboratory, a bigenic mouse model was generated by crossing MMTV-iFGFR1 mice with MMTV-Wnt1 mice. The resulting mouse model showed accelerated tumourigenesis that was demonstrated to depend on the activation of protein translational pathways resulting from cooperation between the FGF and Wnt pathways. Interestingly, the discovered "protein translational gene signature" correlated well with the signature of FGFR1- or FGFR2-overexpressing human breast cancer samples (Pond *et al.*, 2010).

A similar drug-inducible system has been established in prostate cancer cell lines derived from the experimental TRAMP prostate cancer model (Foster *et al.*, 1997). Freeman *et al*. (2003) demonstrated that iFGFR1 was able to promote tumourigenesis and early growth, but iFGFR2 was shown to interfere with these events. Again, the activation of iFGFR1 was shown to cause a higher and longer lasting ERK phosphorylation than activation of iFGFR2 (Freeman *et al.*, 2003), further supporting the importance of the amplitude and duration of ERK activation in determining the response to FGFR activation.

In spite of the highly important knowledge that has been achieved from these drug-inducible and conditional activation systems of FGFR expression described above, it must be taken into consideration that iFGFR transgenes contain several differences from the endogenous FGFRs that may alter their activation kinetics and cellular localisation. Thus, other approaches in studying differential FGFR signalling in breast cancer would be desirable.

2.2.5 Negative modulation of FGF signalling

The wide range of biological effects of FGFs and the variety of the activated signalling pathways imply that FGF signalling must be tightly regulated with regard to timing, duration and signalling strength. Thus, attenuation and negative feedback control of FGFRs are highly important for maintaining the normal function of this signalling system. FGF binding to FGFR leads to ubiquitinylation and rapid internalisation of FGF/FGFR complexes, followed by sorting to recycling or to lysosomal degradation of the complex. The details of these events are only partially known, but at least the receptor ubiquitinylation and internalisation have been shown to require the kinase activity of the receptor (Sorokin *et al.*, 1994; Haugsten *et al.*,

2008) and to involve FRS2 and C3 ubiquitin ligase (Cbl) (Wong *et al.*, 2002; Cho *et al.*, 2004). The level of ubiquitinylation is proposed to play an important role in determining the destination of the complex. For example, FGFR4, which is shown to be less ubiquitinylated, is recycled to the membrane, whereas more extensively ubiquitinylated FGFR1-3 are usually sorted to lysosomes (Haugsten *et al.*, 2005). However, other factors are also involved in determining whether the receptor is recycled or degraded. Regardless, the rate of internalization of FGF/FGFR complexes and their subsequent fates have a major impact on the duration of a given stimulus.

Other mechanisms to attenuate FGFR signalling include the so-called FGF synexpression group of proteins that are co-expressed with FGFs and are also transcriptionally regulated by FGFs (Furthauer *et al.*, 2001; Niehrs and Meinhardt, 2002; Tsang *et al.*, 2002). By inhibiting FGF signalling they establish negative feedback loops that have an important role in regulating the strength and duration of FGF signalling. Sprouty (Spry) was the first such negative regulator to be isolated (Hacohen *et al.*, 1998). An increasing number of proteins has since been identified, including similar expression to FGFs (Sef) (Furthauer *et al.*, 2002; Tsang *et al.*, 2002) and MAP kinase phosphatase 3 (MPK3, also called Dusp6) (Eblaghie *et al.*, 2003; Tsang *et al.*, 2004). The mechanism of action of these factors includes mainly interfering with the FGF-induced signalling molecules. Briefly, Spry acts at the level of Grb2 and/or at the level of Raf, whereas Sef interacts with FGFR on the membrane and inhibits MEK and ERK activity. MPK3 regulates FGF signalling by dephosphorylating MAPK (rev. in Thisse and Thisse, 2005).

The availability of free FGF ligands is also crucial for FGFR activation and provides another mechanism by which to limit FGFR action. For example, the FGFR5 named also FGFRL is able to bind FGFs and, thus, reduce their binding to cell membrane FGFRs when present (Steinberg *et al.*, 2010). In addition, ECM components and HPSGs are important in the regulation of the amount of free FGFs able to trigger FGFR signalling cascades.

2.2.6 Non-canonical FGF signalling

Whereas FGFs classically transmit their signal through the activation of FGFRs, there is increasing evidence that non-tyrosine kinase receptors (NTKRs) and FGFR-interacting proteins are more important than simply being modulators of FGF signalling. Studying the action of such molecules has led to the theory that there are FGF-induced signalling events that bypass the classic FGF-FGFR interaction. It has long been known, that HSPGs are required for a functional FGF-FGFR complex formation (Ornitz, 2000). However, HSPGs are generally considered to be low affinity receptors for FGFs or co-receptors for FGFRs that do not transmit biological signals by themselves. The syndecans are a four-member family of HSBGs that have a well-documented role in facilitating FGF-FGFR complex formation and enhancing FGF signalling (rev. in Murakami *et al.*, 2008). Recent reports show that syndecans have

also signalling capabilities resulting from their ability to bind and activate proteins in the cytoplasm. Syndecan-4 has been shown to activate and control the location of PKCα (Horowitz *et al.*, 2002; Murakami *et al.*, 2002; Keum *et al.*, 2004), regulate Rac activity during cell migration (Tkachenko *et al.*, 2004), and regulate the endocytosis of FGFs (Sperinde and Nugent, 2000). The question of whether all of these responses are trigged by FGFs is still under investigation, but *in vitro* studies indicate that syndecans do have FGF-induced effects independent of FGFRs.

Another example of non-canonical FGF signalling comes from recent reports regarding the neural cell adhesion molecule (NCAM). NCAM is a membrane-bound glycoprotein expressed mainly on the surface of neuronal and glial cells. The interplay of NCAM and FGFR was shown to be important for the neurite outgrowth (Williams *et al.*, 1994) and NCAM-FGFR association was later shown to be important in nonneural cells (Cavallaro *et al.*, 2001; Sanchez-Heras *et al.*, 2006). During the past few years, NCAM has been shown to play a major role in regulating FGF-FGFR interactions by acting as a non-FGF ligand for FGFR that causes the negative regulation of the cellular response to FGF (Francavilla *et al.*, 2007) and/or to the induction of a specific set of FGFR-dependent intracellular events (Francavilla *et al.*, 2009). Interestingly, in the latter study, NCAM induced the internalization of FGFR1, and unlike FGF ligands, it promoted the receptor recycling back to the cell surface, which resulted in sustained FGFR1 signalling.

2.2.7 FGF/FGFRs during mammary gland development

The mammary gland develops in two distinct phases, embryonic and postnatal. While postnatal development and functional differentiation are regulated predominantly by systemic steroid and peptide hormones in the reproductive phase of life, the embryonic development is initiated by the interaction between the epithelium and the surrounding mesenchyme independently of hormonal regulation. Despite the differences regarding the role of hormones in these different developmental stages, the paracrine interactions between the different tissue compartments plays crucial role throughout mammary gland development (Robinson *et al.*, 1999). In particular, the process of ductal morphogenesis at puberty, when the mammary tree is generated by extensive epithelial cell proliferation, is thought to essentially depend on locally produced growth factors. Studies on the developing murine mammary gland have implicated an important role for FGF-FGFR signalling in early development as well as in the later differentiation and maintenance of the organ. Several FGFs and FGFR1 are expressed during the ductal morphogenesis, and their expression is generally decreased during pregnancy and lactation (Chodosh *et al.*, 2000). A more specific role of FGF-FGFR family members has been demonstrated during the embryonic development of the mammary gland, as deletion of either the FGFR2IIIb or FGF10 genes leads to the complete absence of mammary glands due to the inability to form mammary placodes, which are epithelial structures that invade to the mesencyhme and initiate the formation of the rudimentary ductal tree (Mailleux *et al.*, 2002). Furthermore, the

attenuation of endogenous FGFR2-IgIIIb signalling in the mammary gland by the inducible and reversible expression of a soluble FGFR2-IgIIIb that acts as a ligand trap showed that FGFR2-IgIIIb signalling plays a critical role during developmental stages of the mammary gland, during which it controls the induction, survival and proliferation of the placodes and the terminal end buds (TEB), structures that correspond to the TDLUs in human mammary gland (Parsa *et al.*, 2008).

2.2.8 FGF/FGFRs in breast cancer

As potent mitogens and regulators of a wide variety of physiological events, it is not surprising that aberrant FGF signalling has been implicated in many human cancers, including breast cancer. Early observations of the oncogenic potential of FGFs in mammary tissue were obtained from mouse models with mouse mammary tumour virus (MMTV). Proviral activation of FGF-3, FGF-4 and FGF-8 was shown to cause the formation of mammary tumours (van Leeuwen and Nusse, 1995). The transforming capacity of FGF-8 and FGF-3 was then further confirmed by using transgenic mouse models (Muller *et al.*, 1990; Daphna-Iken *et al.*, 1998). Recently, a high throughput search for common insertion sites for MMTV identified even more FGF family members (FGF-3,-4,-6,-8 and FGFR2) that were frequently activated by MMTV (Theodorou *et al.*, 2007). Furthermore, inappropriate activation of FGFR1 has been shown to result in mammary hyperplasia and invasive lesions (Welm *et al.*, 2002). Although the data from murine models clearly show the potential of FGF-FGFR signalling to promote mammary gland tumourigenesis, the involvement of FGF-FGFR signalling in human breast cancer has not been that clear. During the past few years, however, there have been convincing reports concerning FGFR1, -2 and -4 genetics and their functions in breast cancer. These studies suggest that FGF-FGFR system plays an important role in at least a fraction of breast cancers and also in the progression to hormone-independent, drug-resistant disease (rev. in Hynes and Dey, 2010). FGFR3, in turn, seems to be rarely mutated or amplified in breast cancer, despite the fact that activation mutatios in this gene are common in other cancers. A summary of the alterations in FGFRs observed in human breast cancers are presented in Table II and are discussed in more detail in the following paragraphs.

2.2.8.1 FGFR1 in breast cancer

In the human genome, the FGFR1 gene is located at the 8p11-12 locus, which is amplified in 8-10% of breast cancers (Courjal *et al.*, 1997; Gelsi-Boyer *et al.*, 2005; Elbauomy Elsheikh *et al.*, 2007). The correlation between amplification and FGFR1 expression level has been examined in several studies, with some studies finding correlation whereas others did not (Ray *et al.*, 2004; Gelsi-Boyer *et al.*, 2005; Elbauomy Elsheikh *et al.*, 2007; Bernard-Pierrot *et al.*, 2008). However, when amplified and over-expressed, FGFR1 is associated with poor prognosis and can be considered as an independent prognostic factor for overall survival (Gelsi-Boyer *et al.*,

2005; Elbauomy Elsheikh *et al.*, 2007). A recent report by Turner *et al.* showed strong evidence that FGFR1 over-expression related to FGFR amplification is a key contributor to poor prognosis in luminal-type breast cancers and, furthermore, that FGFR1 over-expression correlated to tamoxifen resistance (Turner *et al.*, 2010b). FGFR1 has a clear tumour growth-promoting role in prostate cancer (Acevedo *et al.*, 2009), thus supporting a strong pro-tumourigenic role for FGFR1 in both of these hormone-related cancers.

2.2.8.2 FGFR2 in breast cancer

Whereas FGFR1 seems to clearly promote cancer progression, the role of FGFR2 in tumour development and progression appears to be more complex. Gene amplification in primary breast cancer has been shown to lead to ligand-independent, constitutive activation of FGFR2 signalling cascades (Adnane *et al.*, 1991; Katoh and Katoh, 2003; Moffa and Ethier, 2007). A missense mutation linked to primary breast cancer has also been found (Stephens *et al.*, 2005). Moreover, accumulation of single nucleotide polymorphisms (SNPs) in intron 2 of the FGFR2 has been found to be associated with breast cancer in two genome-wide association studies of breast cancer patients (Easton *et al.*, 2007; Hunter *et al.*, 2007). The Easton study found one SNP in patients with a strong family history of breast cancer, and it was later shown to correlate more with ER-positive, lower grade and node negative tumours (Garcia-Closas and Chanock, 2008). Furthermore, the SNP found in the Easton study was shown to alter the Oct-1/Runx2 binding site *in vitro*, which led to increased transcription of FGFR2 (Meyer *et al.*, 2008). This result suggested that the increased risk is linked to the increased expression of FGFR2. In accordance with these findings, FGFR2 over-expression has been associated with luminal A –type breast tumours (Nordgard *et al.*, 2007). Recently, FGFR2 amplification and over-expression was also shown to occur in 4% of triple negative breast tumours, and FGFR2 expression was found to be essential for the growth of FGFR2-amplified cell lines (Turner *et al.*, 2010a). In addition to genetic alterations, the upregulation of FGFR2 has been reported in breast cancer (Tozlu *et al.*, 2006). FGFR2 mutations have also been found in several other cancers, including gastric, lung and endometrial cancers (Katoh, 2009). Interestingly, FGFR2 loss-of-function mutations have been reported in human melanoma (Gartside *et al.*, 2009), and there is evidence that FGFR2 inhibits, rather than promotes, the initiation and progression of prostate cancer in experimental models (Feng *et al.*, 1997; Freeman *et al.*, 2003). Moreover, FGFR2 has been reported to belong to a set of genes that is downregulated in a gene expression signature associated with poor prognosis for cancer patients, further suggesting a role for FGFR2 as a tumour suppressor (Glinsky *et al.*, 2005). As was discussed in section 2.2.2.1, the alternative splicing of FGFR2 leading to isoforms with very distinct functions may at least partially explain the diversity in the reports regarding the role of FGFR2 in cancer.

2.2.8.3 FGFR4 in breast cancer

The amplification and elevated expression of FGFR4 in breast cancer specimens was first observed several years ago (Jaakkola *et al.*, 1993; Penault-Llorca *et al.*, 1995). More recent reports showed that the upregulation of FGFR4 might be due to a SNP causing a Gly388Arg conversion in the FGFR4 gene, resulting in the generation of a risk variant called FGFR4-R388 (Bange *et al.*, 2002). Interestingly, the same SNP has been linked to a poor response to chemotherapy (Thussbas *et al.*, 2006) and endocrine therapy (Meijer *et al.*, 2008) in breast cancer cohorts. An activating FGFR4 mutation has so far only been found in the MDA-MB453 breast cancer cell line (Roidl *et al.*, 2010). A study from the same authors showed, however, that DNA-damaging agent resistant clones of this particular cell line have upregulated FGFR4 (Roidl *et al.*, 2009), further supporting the idea that FGFR4 plays a role in the development of drug resistance. Mechanistic data concerning FGFR4 function in cancer invasion were published very recently (Sugiyama *et al.*, 2010a; Sugiyama *et al.*, 2010b). In these reports, breast and prostate tumours expressing the FGFR4-R388 risk variant were shown to co-express FGFR4 and membrane type 1 matrix metalloproteinase (MT1-MMP) at elevated levels. Elevated MT1-MMP, in turn, was shown to be responsible for the increased invasiveness and growth of prostate cancer cell lines with the FGFR4 risk variant (Sugiyama *et al.*, 2010b). Furthermore, the FGFR4-R388 variant was shown to induce MT1-MMP phosphorylation and endosomal stabilisation, which increased FGFR4 autophosphorylation. In contrast, the FGFR4-G388 variant down-regulated MT1-MMP (Sugiyama *et al.*, 2010a). These data strongly suggest that FGFR4 polymorphisms can be a crucial factor in ECM degradation and cancer invasion.

2.2.9 FGF expression in breast cancer

As mentioned above, several FGFs have been demonstrated to drive tumourigenesis in the murine mammary gland. However, there are limited corresponding observations of FGF overexpression in human breast cancer specimens. FGF-4 is located within the 11q13 genetic locus, which is commonly amplified in about 18% of breast cancers (Karlseder *et al.*, 1994), but there are no data concerning its possible overexpression. FGF-3 is located in the same amplicon as FGF-4, and its amplification in turn has been shown to correlate with increased aggressiveness in node-negative breast carcinoma (Fioravanti *et al.*, 1997). Increased expression of FGF-8 (Marsh *et al.*, 1999) and FGF-10 (Theodorou *et al.*, 2004) has been reported in human breast cancer samples. Data concerning FGF-1 and FGF-2 expression in breast cancer are ambiguous. Some studies have shown low FGF-2 expression to be linked with poor prognosis in breast cancer patients (Colomer *et al.*, 1997) or have shown levels to be lower in breast cancer than in normal tissue (Luqmani *et al.*, 1992), whereas others have found that elevated FGF-2 is linked with a more aggressive form of the disease and that this protein is elevated in the sera of breast cancer patients (Sliutz *et al.*, 1995; Visscher *et al.*, 1995). Similarly, FGF-1 has been reported to be both reduced (Bansal

et al., 1995) and elevated (Yoshimura *et al.*, 1998) in breast cancer cells. In addition, high FGF-2 levels have been shown to correlate with drug resistance in different types of human tumours (Miyake *et al.*, 1998b; Gan *et al.*, 2006). Of the other FGF family members, some (FGF-5, FGF-7, FGF-9) are expressed or induced by certain treatments in breast cancer cell lines (Lyakhovich *et al.*, 2000; Hanada *et al.*, 2001; Perera *et al.*, 2008), but no reports on their expression in breast cancer have been published.

Table II: Dysregulation and/or genetic alterations of FGFRs in human breast cancer (BCa)

FGFR	ALTERATION	EFFECT	REFERENCES
FGFR1	Amplification of 8p11-12 locus in 8-10% of Bca	Over-expression of FGFR1 leads to increased growth and survival of cancer cells. Association with Tam resistance in luminal breast cancers.	(Elbauomy Elsheikh *et al.*, 2007; Turner *et al.*, 2010b)
FGFR2	Amplification of 10q26 locus in 4-12% of BCa, and in 4% of TNBCs	Possible over-expression	(Adnane *et al.*, 1991; Turner et al., 2010a)
	SNPs in intron 2 of FGFR2 gene	SNPs are associated with increased risk for ER positive BCa. One of the SNPs alter transcription factor binding site leading to increased expression of FGFR2.	(Easton *et al.*, 2007; Hunter *et al.*, 2007; Garcia-Closas and Chanock, 2008; Meyer *et al.*, 2008)
FGFR4	Over-expression	Association with resistance to endocrine- and chemotherapy	(Meijer *et al.*, 2008; Roidl *et al.*, 2009)
	Gly388Arg SNP	Leads to increased expression of FGFR4, association with Tam resistance, increased ECM degradation and invasion	(Bange *et al.*, 2002; Thussbas *et al.*, 2006; Sugiyama *et al.*, 2010a; Sugiyama *et al.*, 2010b)

2.2.9.1 FGF-8

Based on their sequence similarity, FGF-8, FGF-17 and FGF-18 form a subfamily within the FGF protein family. In addition to having similar gene structure, these FGFs show overlapping expression patterns and receptor-binding specificities (Ornitz and Itoh, 2001). FGF-8, -17 and -18 have an important role during embryogenesis, and they are generally expressed at a low level in adult tissues. In particular, the expression of FGF-8 and FGF-17 is highly restricted and spatially coordinated in the developing embryo (Maruoka *et al.*, 1998). Interestingly, FGF-8 was shown to induce FGF-17 expression in zebrafish embryos (Reifers *et al.*, 2000) and a similar effect was also demonstrated in prostate cancer cell lines (Heer *et al.*, 2004). FGF-8, -17 and -18 have transforming capability in NIH3T3 cells and are generally considered as foetal-oncogenes (Hu *et al.*, 1998; Xu *et al.*, 1999).

FGF-8 was originally cloned as an androgen-induced growth factor (AIGF) from the mouse mammary tumour cell line, SC-3 (Shionogy carcinoma -3) (Tanaka *et al.*, 1992). When studied further, it was identified as an FGF and was found to be essential to gastrulation and morphogenesis in mouse embryos; an FGF-8 knockout (KO) was shown to be lethal at an early stage (E9.5) of embryogenesis (Ohuchi *et al.*, 1994; Sun *et al.*, 1999). Heterozygous FGF-8 KO mice with variable levels of FGF-8 in tissues in turn had cardiac and neural defects, abnormal left-right axis specification, hypoplastic pharyngeal arches, abnormal craniofacial development and small or absent thymuses (Abu-Issa *et al.*, 2002). Furthermore, conditional FGF-8 KO mice have shown FGF-8 to be important in craniofacial and limb development (Trumpp *et al.*, 1999; Lewandoski *et al.*, 2000). In adult mice, the strongest FGF-8 expression is detected in ovaries and testes (Fon Tacer *et al.*, 2010). There is increasing evidence that FGF-8 is involved also in bone biology. FGF-8 has been shown to induce osteoblast proliferation and differentiation *in vitro* (Valta *et al.*, 2006; Lin *et al.*, 2009), and contribute to the bone metastasis of prostate cancer (Valta *et al.*, 2008).

In human adult tissues, FGF-8 is expressed at a low level. Interestingly, its expression is mainly found in hormone-regulated tissues such as the kidney, breast, prostate and testis (Ghosh *et al.*, 1996; Tanaka *et al.*, 1998; Marsh *et al.*, 1999). In addition, FGF-8 (and FGF-17 and -18) has been detected in human peripheral blood leukocytes and bone marrow samples from healthy donors, which indicates that FGF-8 may have a role in normal haematopoiesis (Nezu *et al.*, 2005). Although FGF-8 expression is detected at a low level in normal hormonally-regulated organs, increased expression has been observed in breast, prostate and ovarian cancer samples (Dorkin *et al.*, 1999; Marsh *et al.*, 1999; Valve *et al.*, 2000), suggesting a role in hormone-regulated cancers. In prostate cancer, the expression of FGF-8 has been correlated to tumour progression and a poor prognosis (Dorkin *et al.*, 1999; Darby *et al.*, 2006). In addition, FGF-8 and VEGF have been shown to be co-expressed in prostate cancer, which may lead to synergistic effects on tumour angiogenesis (West *et al.*, 2001). Several breast and prostate cancer cell lines express FGF-8, further suggesting a role in cells that have undergone malignant transformation (rev. in Mattila and Harkonen,

2007). Recently, mechanisms behind the ability of FGF-8 to promote breast tumour growth was suggested to include increased CyclinD1 production reflecting the activation of cell cycle machinery, and the protection from apoptosis in mouse S115 and human MCF-7 cells (Nilsson *et al.*, 2009). A summary of the previous findings of FGF-8 mediated effects in breast and prostate cancer experimental both in *in vitro* and *in vivo* models is presented in Table III.

The first of the three exons in the FGF-8 gene is alternatively spliced which results in eight different isoforms in the mouse (a-h) and four in humans (a, b, e, f) (Crossley and Martin, 1995; Gemel *et al.*, 1996). The alternatively spliced FGF-8 isoforms differ in their N-termini but are identical in their C-terminal domains. The functional role of the different isoforms is still largely unknown, but the isoforms differ in their transforming potential and expression patterns. FGF-8b has been shown to be the most transforming isoform and has a strong angiogenic potential (MacArthur *et al.*, 1995a; Ghosh *et al.*, 1996; Mattila *et al.*, 2001a; Ruohola *et al.*, 2001). During development, FGF-8a and -b have distinct roles in the brain development where they regulate the expression pattern of En2, Otx2 and Gbx2, which are essential for cerebellar differentiation (Sato *et al.*, 2001).

In terms of receptor binding, FGF-8 bind with high affinity to FGFR1-3 IgIIIc forms and to FGFR4 (MacArthur *et al.*, 1995b; Ornitz *et al.*, 1996; Olsen *et al.*, 2006), which means that the targets for FGF-8 signalling are likely to be localised mainly in the mesenchymal/stromal cells. Moreover, FGF-8 isoforms are reported to bind FGFR2IgIIIc receptors with different affinities, which could also explain their functional differences (Olsen *et al.*, 2006). Interestingly, the HSPG Syndecan-1 has been shown to play an inhibitory role in the FGF-8b –induced activation of FGFRs in S115 breast cancer cells (Viklund et al., 2006).

Although it was discovered as an androgen induced factor, androgen regulation of FGF-8 is still under discussion. In Shionogi carcinoma 115 (S115) and SC-3 cell lines, which are derived from the same mouse mammary carcinoma (Minesita and Yamaguchi, 1965), FGF-8 is considered to be androgen-induced through the insertion of steroid hormone-sensitive LTR promoter elements of MMTV in the vicinity of the FGF-8 gene (MacArthur *et al.*, 1995c; Valve *et al.*, 2001). FGF-8 was later shown to be androgen responsive in S115 cells due to a translocation to a chromosomal region that contains androgen-regulated genes (Erdreich-Epstein *et al.*, 2006). In either case, these reports suggest that androgen inducibility, at least in the S115 model, is not inherent in the FGF-8 gene. However, FGF-8 has also been reported to be secreted in response to androgens in MDA-MB231 human breast cancer cells (Payson *et al.*, 1996), and in LnCap prostate cancer cells the FGF-8 promoter is shown to contain an androgen response element (ARE) which leads to androgen-regulated expression (Gnanapragasam *et al.*, 2003). Altogether, there is still no consensus about whether FGF-8 transcription is under direct androgen regulation. However, a relationship between androgen signalling and FGF-8 in breast cancer has also been suggested by a

study in which BRCA1-mutated breast tumours significantly lacked both AR and FGF-8 expression when compared to sporadic breast tumours (Berns *et al.*, 2003).

Table III. Tumour growth-promoting effects of FGF-8 in breast and prostate cancer experimental models

EFFECT	**SUGGESTED MECHANISM**	**MODEL WITH FGF-8 STIMULATION OR OVER-EXPRESSION**	**REFERENCES**
Altered cell morphology	Regulation of target genes	SC-3, S115, MCF-7 *in vitro*	(Tanaka *et al.*, 1992; Mattila *et al.*, 2001b; Ruohola *et al.*, 2001)
Increased proliferation of cancer cells *in vitro* and *in vivo*	MAPK, PI3K and p38 activation, increased CyclinD1 and B1 expression, regulation of other target genes	S115, MCF-7, PC-3, LnCap *in vitro* and/or *in vivo*	(Tanaka *et al.*, 1995; Song *et al.*, 2000; Mattila *et al.*, 2001a; Ruohola *et al.*, 2001; Nilsson *et al.*, 2009)
Increased invasion	Increased MMP-9 activity, regulation of other target genes	MCF-7 *in vitro*	(Ruohola *et al.*, 2001)
Increased angiogenesis in tumours	Increased endothelial cell proliferation and migration, altered production of angiogenic factors from tumour/stromal cells	S115, MCF-7, PC-3 *in vivo*, CAM	(Mattila *et al.*, 2001a; Ruohola *et al.*, 2001; Valta *et al.*, 2009b)
Protection from apoptosis	PI3K pathway activation	S115, MCF-7 *in vitro*	(Nilsson *et al.*, 2009)
Role in bone metastasis	Stimulating effects on bone cells and osteoprogenitor cells, regulation of bone metastasis related genes in tumour cells	PC-3 *in vitro*, *in vivo*	(Valta *et al.*, 2006; Valta *et al.*, 2008; Valta *et al.*, 2009a)
Induction of prostate tumorigenesis	Dedifferentiation of the epithelium, formation of activated stroma, regulation of target genes	ARR_2PB–FGF-8b transgenic Mouse	(Song *et al.*, 2002; Elo *et al.*, 2010)

2.2.9.1.1 FGF-8 target genes in cancer

Until recently, there have been few studies on the specific target genes of FGF-8. During limb development, FGF-8 has been shown to regulate the expression of MKP3, a known negative regulator of FGF signalling (Kawakami *et al.*, 2003). In the same study FGF-8 was shown to differentially activate ERK and PI3K pathways in mesenchymal and ectodermal cell compartments. Recently, FGF-8-regulated genes have been recognised from FGF-8-overexpressing PC-3 prostate cancer cells (Valta *et al.*, 2009a) and from prostate-specific FGF-8b transgenic mice (Elo *et al.*, 2010). Many of the FGF-8-induced genes found in these studies have previously been documented or predicted to have roles in processes such as cell growth, angiogenesis (CCL2 and DDAH2), proliferation (CRIP1 and SHC1), development, and bone metastasis (OPN and SPARC). Osteopontin (OPN) is a phosphoprotein that is secreted at elevated levels by several types of tumour cells, including breast cancer cells. Previously, OPN has been reported to be linked to FGFR1 signalling in different cell types (Li *et al.*, 2002; Freeman *et al.*, 2003).

2.2.10 FGFs in breast cancer angiogenesis

An angiogenic heparin-binding protein fraction originally isolated from endothelial cells in the 1970s (Folkman *et al.*, 1971) was later shown to contain FGF-1 and FGF-2, the two prototypic FGFs. Since then, these factors have been demonstrated to have angiogenic properties in numerous studies and, thus, they are generally considered angiogenic FGFs. FGFs are thought to mediate their angiogenic effects by modulating endothelial cell proliferation, migration, protease production, integrin and cadherin receptor expression, and intercellular gap junction communication (Javerzat *et al.*, 2002). In addition to FGF-1 and FGF-2, FGF-8 has been shown to act as an angiogenic factor in experimental systems by inducing endothelial cell proliferation, migration, the formation of tubule-like structures and capillary sprouting in a chicken chorion allantoic membrane (CAM) assay (Mattila *et al.*, 2001a). Furthermore, FGF-8 has been shown to induce a highly vascular phenotype in tumours when over-expressed in several cancer cell lines (Mattila *et al.*, 2001a; Ruohola *et al.*, 2001; Valta *et al.*, 2009a). Among the FGFRs, FGFR1 and FGFR2 are considered as responsible for the direct effects on the endothelial cells, whereas FGFR3 or FGFR4 are not expressed in the endothelium (Presta *et al.*, 2005). Besides having direct effects on endothelial cells, it is probable that FGFs regulate angiogenesis indirectly by regulating pro- and antiangiogenic factors in tumour cells, which then contribute to the angiogenic balance in the tumour microenvironment.

2.3 TARGETING GROWTH FACTOR SIGNALING IN BREAST CANCER

As a mediator of both hormonal and auto/paracrine growth signals in breast cancer, growth factor signalling is one of the major focuses for the development of new targeted therapies in breast cancer. Extensive research has been conducted to identify and test compounds that could be suitable for therapeutic use. These efforts have led to a tremendous amount of *in vitro* data and to increasing numbers of ongoing clinical studies. As discussed in the previous paragraphs, almost all of the most studied growth factor pathways, such as the IGF-, EGF-, and TGFβ pathways, and the angiogenic signalling systems are considered potential targets for cancer therapeutics. Here, only a few examples and some recent concerns in the field are discussed along with a more detailed description of FGFR inhibition.

A good example of a successful targeted therapy is the Her2-targeting monoclonal antibodies, such as Herceptin, which were discussed in section 2.1.4.2.1. However, resistance to Herceptin is a major problem in the treatment of Her2-positive metastatic breast cancers. In the case of angiogenesis inhibitors, major concerns have been raised in light of recent results from studies using experimental models of different types of cancers and VEGFR inhibitors that are already in clinical use. These inhibitors have been shown to cause a permanent switch to a more invasive and metastatic tumour phenotype, even though they are still effective in blocking angiogenesis and primary tumour growth (Ebos *et al.*, 2009; Paez-Ribes *et al.*, 2009). Thus, angiogenesis inhibition has now been implicated as a driving force in tumour progression, which of course raises serious questions as to wether similar phenomenon could occur in human tumours.

2.3.1 FGFR inhibitors as therapeutic agents

Since it has become evident that the FGF/FGFR system is dysregulated in many types of cancers, there is increasing interest in developing therapeutics that target FGFR signalling pathways. There are different approaches to target FGFR signalling, including the use of synthetic FGFR tyrosine kinase inhibitors (TKIs), blocking antibodies, FGF ligand traps that prevent functional FGF-FGFR complexes to form, and finally recombinant FGFs used to stimulate FGFRs in some special conditions (rev. in Turner and Grose, 2010). Several small molecule TKIs are at different stages of clinical development, but so far, only a few studies are published. Most of the *in vitro* reports on TKIs describe compounds that are referred to as "multitargeted" because they also block other receptor tyrosine kinases (RTKs), such as PDGF and VEGFR family members. Examples of such TKIs are PD173074, TKI168 and BMS-5862664. PD173074 is often referred to as a selective inhibitor of FGFRs with an IC_{50} of ~25 nM, whereas it effectively blocks VEGFR2 only at a four-fould higher concentration, which was reported in the original paper (Mohammadi *et al.*, 1998). Nevertheless, PD173074 is used only *in vitro* and in experimental models, where it is shown to inhibit cancer cell proliferation, angiogenesis and tumour growth (Koziczak

et al., 2004; Buchler *et al.*, 2007; Turner *et al.*, 2010a). TKI168 has been recently shown to be a more selective FGFR inhibitor in breast cancer cells and to effectively inhibit mammary tumour growth (Dey *et al.*, 2010). In a phase I clinical study, it caused a partial response in only a small portion (2/35) of cancer patients with advanced solid cancers (Sarker *et al.*, 2008). Because of the problems in simultaneously targeting several RTK families, more selective FGFR inhibitors are being developed, but no data on their efficacy or toxicity are available yet. Side-effects can also be expected with the selective FGFR inhibitors. In particular, the endocrine FGFs (FGF-19, -21 and -23) are important in regulating the metabolism and tissue calcification (Shimada *et al.*, 2004), so it is quite likely that inhibiting these FGFs will result in significant side effects.

In summary, targeting RTKs in the tumour microenvironment has turned out to be complex, and disturbing the existing balance of signalling pathways may result in some unexpected effects. In other words, the blockade of any particular type of cell-surface receptor might create selection pressure that can then promote the survival of those tumour cells that can compensate for blockade by increased signalling through some alternate receptors. However, it is clear that inhibiting the growth factor pathways is indeed effective in preventing cancer progression. Interfering with these pathways may lead to better therapies. When thinking of the concept of the hallmarks of cancer, including limitless replicative potential, self sufficiency of growth signals, resistance to apoptotic signals, angiogenesis, tissue invasion and metastasis and insensitivity to anti-growth signals (Hanahan and Weinberg, 2000), it is probable that simultaneously targeting several of these capasities or by targeting the common downstream signalling molecules could be a key to achieving improvements in therapies and the increased survival of cancer patients.

3 AIMS OF THE PRESENT STUDY

The present study aimed to clarify the mechanisms by which FGF-8 and androgen regulate growth and angiogenesis in breast cancer and to study the differential roles of FGFRs in tumour growth. The specific aims were as follows:

1. To find androgen- and FGF-8-regulated genes in S115 breast cancer cells

2. To characterise FGF-8 signal transduction in S115 cells and to clarify the pathways involved in TSP-1 repression

3. To study the role of the differential signalling of FGFRs 1-3 in breast cancer cells and in nude mouse tumours *in vivo*

4 MATERIALS AND METHODS

4.1 REAGENTS

DMEM, L-glutamine, 17β-estradiol (E2), cycloheximide (CHX) and flutamide were purchased from Sigma (St. Louis, MO, USA), MEBM from Lonza (Basel, Switzerland), and FBS was purchased from Life Technologies, Inc. (Paisley, Scotland, UK). U0126, PD98059, LY294002, were from Cell Signaling technologies (Beverly, MA, USA). PD173074 was a gift from Pfizer (Ann Arbor, USA). Testosterone (Te, 4-androsten-17-ol-3-one) was from Sigma. Antibodies for P-ERK, ERK, P-p38, p38, P-Akt, Akt, and P-FRS2α were from Cell Signaling technologies; the β-actin antibody was from Sigma. The HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were from Jackson ImmunoResearch (West Grove, PA, USA). Western blotting PVDF membranes were from Millipore (Billerica, MA, USA). The enhanced chemiluminescence (ECL) detection system was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), and size markers for western blotting were from BioRad (Hercules, CA, USA). Recombinant mouse and human FGF-8 and FGF-7 were obtained from R&D Systems (Minneapolis, MN, USA) and FGF-2 was purchased from Sigma (I) or from R&D Systems (II). Trizol reagent was from Invitrogen (Carlsbad, CA, USA). The RNeasy RNA extraction kit and SYBR green PCR Master Mix were purchased from Qiagen (Valencia, CA, USA).

4.2 CELL CULTURE (I-III)

4.2.1 Cell lines

The parental S115 mouse mammary tumour cells (Darbre and King, 1988) were maintained in DMEM supplemented with 4 % heat-inactivated FBS (iFBS) and 10 nM testosterone. G418 (300 μg/ml) and puromycin (3 μg/ml) were used as selection antibiotics for transfected S115-Mock/FGF8b lines (Mattila *et al.*, 2001a) and shRNA expressing sh-S115 cells (shLacZ,shR1,shR2,shR3), respectively. The MCF10A cell line was cultured in the MEBM medium containing all SingleQuot additives that were supplied with the MEGM Bullet Kit (Lonza). The human MCF-7 cells were maintained in RPMI 1640 culture medium supplemented with 10% iFBS, 2 mM L-glutamine, insulin (4 μg/ml) and 1 nM 17β-estradiol (E2).

4.2.2 Inhibition of protein synthesis in vitro (I)

To study the effect of inhibition protein synthesis on TSP-1 regulation by Te, the cells were treated with 10 μg/ml cycloheximide (CHX) for two hours prior Te stimulation. The cells were lysed and the RNA extracted after 6, 12 or 24 hours of Te treatment.

4.2.3 Use of protein kinase inhibitors in vitro (II,III)

The protein kinase inhibitors U0126 (10 µM), PD98059 (10 µM), SB203580 (10 µM), LY294002 (10 µM), and PD173074 (1 µM), all of which were prepared in DMSO, were added in 4% DC-FBS (dextran charcoal-treated fetal bovine serum)-DMEM to cells 1-2 hours prior to FGF-8 stimulation.

4.2.4 Te and FGF stimulation in vitro (I-III)

For the analysis of TSP-1 mRNA expression, S115 cells were cultured in DMEM supplemented with 4% DC-FBS. After being deprived of Te for three days, the cells were challenged with 10 nM Te, 10 ng/ml of rmFGF8b or 10 ng/ml of rhFGF2 for the indicated periods. Flutamide (Flut; 1 µM) was used as an antiandrogen.

For the analysis of TSP-1 mRNA expression and protein phosphorylation in MCF10A cells, the cells were plated subconfluently in full medium on 3.5 cm plates. After 24 hours, the cells were rinsed with MEBM and the medium was changed to MEBM containing hydrocortisone (0,5 µg/ml). Twenty-four hours later, cells were challenged with 25 ng/ml rmFGF-8 in MEBM in the presence of hydrocortisone for the indicated time periods. Cells were cultured in a similar manner for the detection of TSP-1 mRNA. The protein kinase inhibitors or DMSO alone were added one hour prior to FGF-8.

For experiments with FGF-8b, FGF-2 and FGF-7, the shS115 and MCF-7 cells were pre-cultured in DMEM (S115) or RPMI (MCF-7) supplemented with 4% DC-FBS. After depriving cells of Te for 24-48 hours the medium was replaced with Ham's F-12 containing bovine serum albumin (BSA;0.2%) and mouse recombinant FGF-8b (25 ng/ml), FGF-2 (10 ng/ml) or by FGF-7 (100 ng/ml) proteins. The FGFR inhibitor PD173074 was added 30 minutes prior to FGF-8 treatment.

4.2.5 FGFR2 transfection (III)

The FGFR2 expression constructs SC112364 and SC111932 (here, named pFGFR2IgIIIb and pFGFR2IgIIIc, respectively) were purchased from Origene (OriGene Technologies Inc, Rockville, MD, USA). shLacZ cells were transfected with FGFR2IgIIIb or FGFR2IgIIIc plasmids or with the vector control pCMV6-Neo using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Gene and protein expression were studied 24-96 h post transfection by qRT-PCR and western blotting, respectively.

4.2.6 Proliferation assays (III)

The proliferation rate of sh-S115 cells was evaluated by way of [H^3]-thymidine incorporation. Cells were seeded in 96-well plates in full growth medium or, for experiments with FGF-8b and/or PD173074, in pre-culture medium followed by FGF-8b treatment as described above. Cells were incubated with [H^3]-Tthymidine (0.1 µCi/well; Amersham Bioscience Ltd., Bucks, UK) for 2 h, and [H^3]-thymidine incorporation was determined by scintillation counting in Wallac MicroBeta TriLux equipment (Wallac Oy, Turku, Finland). The proliferation rate of the FGFR2-overexpressing cells was measured by WST-1 assay (Roche, Basel, Switzerland) according to the manufacturer's instructions

4.3 RNA METHODS

4.3.1 RNA extraction (I-III)

Total RNA for northern blotting was extracted by the guanidium isothiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). RNA for the qPCR analysis of the cell lines was extracted and treated by DNAse using the RNeasy RNA extraction kit according to the manufacturer's instructions (Qiagen). Total RNA from mouse tumours was extracted using Trizol reagent (Invitrogen) according to manufacturer's instruction. After Trizol extraction, tumour RNA was further purified and DNase-treated with RNeasy (Qiagen) according to the manufacturer's RNA clean-up procedure.

4.3.2 RNA quantitation methods (I-III)

4.3.2.1 Northern analysis (I)

Twenty micrograms of total RNA were electrophoresed in 1 % agarose gels containing formaldehyde, stained with ethidiumbromide (EtBr), photographed under UV light, and blotted to Gene Screen Plus nylon membranes (NEN Research Products, Boston, MA) using standard conditions. Inserts for FGF-8 (Ruohola *et al.*, 1995), VEGF (Leung *et al.*, 1989) and TSP-1 were used as hybridiation probes. The TSP-1 insert (600 bp) was produced by RT-PCR, after which it was purified and confirmed by sequencing. The sequences of the primers used were as follows: 5'-CAGGTCGATGAGTGCAAAGA-3' and 5'-GTCTGCTTGGTCAGGGTTGT-3'. Hybridisation with a 28S cDNA probe (*Sal*I-*Eco*RI fragment of mouse S28) was used as a reference. To study the effect of FGF8 antisense oligonucleotides on TSP-1 expression, a filter containing poly(A)$^+$RNA from S115 cells treated with FGF8 antisense or sense phosphorothioate oligonucleotides in the presence of Te (Mattila *et al.*, 2001a) was hybridised with TSP-1. These phosphorothioate oligonucleotides have

been designed to encompass the translation initiation site of FGF-8 and have been shown to downregulate FGF8 mRNA expression in S115 cells (Mattila *et al.*, 2001a). Hybridisation of the blot with [^{32}P]end-labelled oligo(dT)$_{15}$ was used as a control for poly(A)$^+$RNA loading. A Microcomputer Imaging Device (MCID M4 Image Analyser, Imaging Research, St. Catherines, Ontario, Canada) was used for densitometric quantitation of the intensities of hybridisation signal intensities.

4.3.2.2 Quantitative real time PCR (II, III)

The cDNA was synthesised by using 0,8-1 µg of total RNA as the starting material. Quantitation of mRNA was performed with a QuantiTect SYBR green real-time PCR kit (Qiagen) using a DNA Engine Opticon system (MJ Research, Inc., Waltham, MA, USA). The primers used are presented in Table IV. The primers used for mouse FGFR1, 2 and 3 were described in (Kurosu *et al.*, 2007). The primers for mouse FGFR1IgIIIb and –IgIIIc were adapted from (Kettunen *et al.*, 1998). The amount of mRNA of the gene of interest was normalised to Cyclophilin B, GAPDH or β-actin expression. The results were analysed using the 2 delta-delta CT method (Livak and Schmittgen, 2001).

Table IV. Gene specific primers used in quantitative RT-PCR

GENE NAME	**FORWARD PRIMER**	**REVERSE PRIMER**
Human TSP-1	5'GGCCTCCCCTATGCTATCAC3'	5'TGCCACAGCTCGTAGAACAG3'
Mouse TSP-1	5'AACTGTGACCCTGGACTTGC3'	5'GGACTGGGTGACTTGTTTCC3'
GAPDH	5'ACCACAGTCCATGCCATCAC3'	5'TCCACCACCCTGTTGCTGTA3'
Human β-actin	5'CGTGGGGCGCCCCAGGCACCA3'	5'TTGGCCTTGGGGTTCAGGGGG3'
Mouse Cyclophilin B	5'TTGGCCTTGGGGTTCAGGGGG3'	5'GAAGCGCTCACCATAGATGC3'
Mouse FGFR2IgIIIb	5'GGACAAGCACGTGGAAAAG3'	5'ACTGGTTGGCCTGCCCTATA3'
Mouse OPN	5'CCCGGTGAAAGTGACTGATT3'	5'TTCTTCAGAGGACACAGCATTC3'
Human FGFR1IgIIIc	5'GTGAATGGGAGCAAGATTGG3'	5'GCAGAGTGATGGGAGAGTCC3'

4.4 WESTERN ANALYSIS (I-III)

4.4.1 Western analysis of secreted TSP-1 (I)

The production of TSP-1 protein was assayed in serum-free conditioned medium (CM) collected from 10^5 parental S115, S115 vector-transfected (S115-m1 and S115-m3) and S115 FGF8b-transfected (S115-b1 and S115-b14) cells after the indicated time points. Media samples were concentrated using Microcon Y-30 columns (Millipore, Germany), diluted in Laemmli sample buffer, separated by sodium dodecyl-

polyacrylamide gel electrophoresis and blotted onto nylon filters (Millipore, Germany) in standard conditions. Coomassie blue -staining of parallel gels was used to confirm the equal loading of proteins. TSP-1 was detected by monoclonal mouse anti-TSP-1 antibody (0.3 μg/ml) (Neomarkers, CA) and a secondary antibody, a HRP-labelled anti-mouse IgG, at a dilution of 1:5000 (Jackson ImmunoResearch Laboratories). Protein bands were visualised using an ECL (Amersham Life Science). A Microcomputer Imaging Device (MCID M4 Image Analyser, Imaging Research, St Catherines, Ontario, Canada) was used for quantitation.

4.4.2 Western analysis of cellular proteins (II,III)

To study the activation of different kinase pathways, the cells were lysed after 0, 5, 10, 20, 30, 120 and 180 min of FGF-8 treatment in Laemmli buffer. For the analysis of FGFR, Cyclin D1 and Cyclin B expression, the cells were cultured in normal growth medium and lysed in Laemmli buffer. The cell lysates were sonicated for 10 sec and boiled for 5 min in the presence of β-mercaptoethanol. The proteins were then separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis and blotted on nylon filters under standard conditions. The membranes were blocked with 8% skim milk in Tris-buffered saline (TBS)/0,1% Tween-20 and incubated with the primary antibodies for P-p44/42, P-Akt, P-JNK1/2, P-MAPKAPK-2, P-FRS2α, FGFR1, FGFR2 or FGFR3 at 4°C overnight and then incubated with anti-rabbit or anti-mouse IgG-HRP secondary antibodies for 1-2 h at room temperature. The protein bands were visualised using ECL. After detecting the proteins of interest, the filters were stripped in TBS-SDS containing β-mercaptoethanol and reprobed with antibodies for total p44/42, Akt, JNK1/2, MAPKAPK-2 or β-actin. An MCID M4 Image Analyser was used to quantify of the protein bands.

4.5 SILENCING OF FGFR EXPRESSION BY shRNAs (III)

4.5.1 shRNA constructs

Bacteria containing the desired shRNA in the pLKO vector (Sigma) were streaked out on ampicillin-containing plates and grown over-night at 37°C. Single colonies were picked and expanded into a miniculture of LB/Amp medium for 10 hours and mini DNA preps were prepared using the PureYield Plasmid Miniprep Kit (Promega) following the manufacturer's protocol. DNA was sent for sequencing with the following primers: 5'- CAAGGCTGTTAGAGAGATAATTGGA-3' and 5'-CTTTAGTTTGTATGTCTGTTGC-3'. After sequence verification, a single colony per shRNA was expanded for six hours in 5 ml LB/Amp and subsequently grown over-night in 500 ml LB/Amp. DNA maxi preps were then prepared using the Nucleobond PC-500 Kit (Macherey-Nagel) or with a DNeasy (Qiagen) according to the manufacturer's respective protocols.

4.5.2 Generation of S115 cells stably expressing shRNAs against FGFR1,2 and 3

LKO.1 shRNA lentiviral vectors were produced by calcium-phosphate mediated co-transfection of 14.5 μg pLKO.1 siRNA, 8.3 μg pCMVΔR8.91 and 2.1 μg pMD.G into 293T cells. Forty-eight hours later, the virus-containing media was collected and filtered through 0.45 μm pore-size filters. The lentiviral titre was determined using MBA-13 cells (Tuittila *et al.*, 2000). The cells were seeded onto 6-well plates at a concentration of 1 x 10^5 cells/well. Twenty-four hours later, diluted viral supernatant was added in the presence of 8 μg/ml polybrene and incubated for six hours. Puromycin (3 μg/ml) was added forty-eight hours post-infection. After eight days, the medium was removed, the cells were fixed and stained with crystal violet and the colonies were counted. The titers were routinely 1-5 x 10^7 cfu/ml.

S115 cells were seeded onto 6-well plates. After 24 hours lentiviral supernatant was added at an moi (multiplicity of infection) 100-300 together with 8 μg/ml polybrene. After incubation at 37°C for 6 hours the transduction medium was replaced with fresh medium and the cells were incubated for 72 hours before puromycin (3 μg/ml) was added. Prior to use, cell media were tested for the absence of replication-competent virus by measuring HIV-1 p24 antigen expression by RETROtek HIV p24 antigen ELISA assay (ZeptoMetrix Corp., NY). The new puromycin resistant pools of S115 cells expressing shRNAs against LacZ, FGFR1, FGFR2 and FGFR3 were named as shLacZ, shR1B, shR1D, shR2IA, shR2ADG and shR3B cells. For simplicity, the cells chosen for further studies based on their FGFR silencing efficiency (shLacZ, shR1B, shR2IA and shR3B) will hereafter be referred to as shLacZ, shR1, shR2 and shR3 cells.

4.6 CANCER CELL INOCULATION TO NUDE MICE (III)

Six-week-old male nude athymic (nu/nu) mice (Harlan, the Netherlands) were maintained under controlled pathogen-free environmental conditions: 20-21ºC, 30-60% relative humidity, a 12-hour lighting cycle, standard rodent pellet diet (SDS, Witham, Essex, UK) and tap water ad libitum. A total of 30 mice were used for this study. Animals were divided into five groups according to their weight. Thirty minutes before the inoculation of tumour cells, mice were subcutaneously injected with an analgesic drug (Temgesic, 0.3 μg/g, Schering-Plough Nv, Brussels, Belgium). S115, shLacZ, shR1, shR2 and shR3 cells were inoculated subcutaneously (1 x 10^6 cells in 100 μL PBS) into the flanks of the mice. The mice were anaesthetised by means of isoflurane inhalation (1.5-3%, air flow 200 ml/min, Univentor 400 anaesthesia unit, Univentor Ltd., Zejtun, Malta). Concomitantly with tumour cell inoculation, the mice were implanted with a 60-d release testosterone pellet containing 10 mg of Te (Innovative Research of America, Toledo, OH) under anaesthesia. Animal welfare was monitored daily. Tumour measurements were performed every 3-4 days with a calliper and the tumour volume was calculated according to the formula of $V=(\pi/6)(d_1 x d_2)^{3/2}$, where d_1

and d_2 are perpendicular tumour diameters (Warri *et al.*, 1993). The animal experiments were carried out according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, Statutes 1076/85 § and 1360/90 of The Animal Protection Law in Finland, and EU Directive 86/609. The experimental procedures were reviewed by the local Ethics Committee on Animal Experimentation at the University of Turku and approved by the local Provincial State Office of Western Finland.

The shLacZ, shR1, shR2 and shR3 tumour-bearing mice were sacrificed four weeks after inoculation. Tumours were exposed by removing the overlaying skin. The final volume of the tumours was measured with callipers and the volume was calculated according to the method described by Janik *et al.*, 1975 as $V = (d_1 \times d_2 \times d_3) \times (\pi/6)$, where d_1, d_2 and d_3 are the three perpendicular tumour diameters. Each tumour was then divided into halfs. One-half was fixed in formalin for histological examination, and the other was frozen in liquid nitrogen for RNA analysis.

4.6.1 Use of PD173074 in vivo

The experiment described above was performed twice. During the second experiment, a group of mice bearing shR2 tumours were treated with the FGFR inhibitor PD173074, beginning two weeks after the inoculation of cells. PD173074 in PBS (25 mg/kg) was administered intraperitoneally once a day, five days/week until the end of the experiment. A control group of shR2 tumour bearing mice were treated similarly with the vehicle (DMSO in PBS).

4.7 IMMUNOHISTOCHEMISTRY (III)

Formalin-fixed paraffin sections of sh-cell tumours (n=6) were deparaffinised with xylene and rehydrated through a graded series of ethanol solutions and a final wash in distilled water. To inhibit endogenous peroxidase activity, the sections were incubated with 3% hydrogen peroxide in methanol for 10 minutes. They were then rinsed three times in distilled water. For PECAM-1 staining, antigen retrieval was achieved with immersion in Tris/EDTA (10 mM Tris Base, 1 mM EDTA, pH 9.0) and heated in a microwave oven for 15 minutes. The slides were left to cool for 20 minutes before being washed with PBS and 0.1% Tween-20 (PBST). Sections were blocked with 1% BSA-PBST containing 10% normal serum (corresponding to the species origin of the secondary antibody) for 30 minutes. Samples were then incubated with PECAM-1 (2.0 μg/ml; Santa Cruz) antibody in 1% BSA-PBST at 4ºC overnight. The slides were rinsed three times in PBST followed by a 30 minutes incubation with biotinylated anti-goat IgG (3.8 μg/ml; Vector Laboratories). They were rinsed three times in PBST and labelled using a Vectastain ABC Kit (Vector Laboratories) according to the manufacturer's instructions. After three rinses in PBS, antibody binding was visualised by incubating the sections in 3,3'-diaminobenzidine (DAB Kit, Vector Laboratories)

for 3-5 minutes. The slides were rinsed in distilled water and, thereafter, in tap water. All sections were counterstained with haematoxylin and coverslips were applied with Mountex mounting medium (Histolab, Sweden).

The sections were viewed and analysed using a 10-40X objective on an Olympus BX60 microscope equipped with a digital camera. The density of PECAM-1-positive vessels in tumours was quantified by counting the lengths in each tumour (three fields per one tumour) by using ImageJ software (ImageJ, 1.37v, Wayne Rasband, National Institutes of Health).

4.8 DETERMINATION OF APOPTOSIS IN TUMOURS (III)

4.8.1 TUNEL assay

Apoptotic cell nuclei in formalin-fixed, paraffin-embedded tumor sections were detected by TUNEL assay with the DeadEnd™ Fluorometric TUNEL system according to the manufacturer's instructions (Promega). Apoptotic cells (fluorescein) and all cells (DAPI) were counted in 3-15 fields per section by 20X objective on an Olympus BX60 microscope.

5 RESULTS

5.1 FGF8-ACTIVATED KINASE PATHWAYS IN S115 BREAST CANCER CELLS AND IN MCF10A BREAST EPITHELIAL CELLS (II)

To study the effect of FGF-8 on some well-known signalling pathways, western blot analysis was performed to detect phosphorylated FRS2α, ERK1/2, p38, Akt, and JNK1/2 proteins after 5-180 min of FGF-8 stimulation in S115 and/or MCF10A cells. The adaptor protein, FRS2α, was rapidly phosphorylated after FGF-8 stimulation in both S115 and MCF10A cell lines, demonstrating that both respond to exogenous FGF-8. In S115 cells, a strong activation of ERK1/2 was detected, peaking at 10 min after FGF-8 addition and attenuating after 60 min of treatment. The p38 kinase was also affected by FGF-8, but an approximately two-fold increase in the p38 phosphorylation status was detected only after 60 min of stimulation. The PI3K target, Akt, in turn was already detected in a strongly phosphorylated state already in the 24 hour serum-starved cells. However, when the conditioned serum-free medium was replaced by fresh medium two hours prior to FGF-8 stimulation, a response in Akt phosphorylation after FGF-8 addition was observed. These results suggest that the PI3K pathway is a target for FGF-8 induced activation, but the effect is masked by the highly active auto- and paracrine signalling that results in constant activity of this pathway in S115 cells. The stress-activated MAPK JNK1/2 was not phosphorylated in response to FGF-8 in this experimental system. In MCF10A cells, activation of ERK1/2 was observed in response to FGF-8, but neither p38 nor Akt phosphorylation levels were affected. Altogether, the response to FGF-8 was weaker in MCF10A cells than in S115 cells in terms of activation of these particular kinases.

5.2 REGULATION OF TSP-1 EXPRESSION IN BREAST CANCER CELLS AND IN BREAST TISSUE (I,II)

5.2.1 Androgen and FGF-8 –induced repression of TSP-1 in S115 cells

TSP-1 was originally recognised as a target gene for androgens in the cDNA expression array performed on S115 cells treated with or without testosterone (Te) (Tarkkonen, Master's Thesis, 2001). Confirmation of this result by northern and western analyses showed that Te strongly represses TSP-1 mRNA expression and protein secretion from S115 cells, and the effect can be blocked by treatment with the anti-androgen, flutamide. When protein synthesis was blocked by cycloheximide, Te was no longer able to repress TSP-1 expression, suggesting the Te effect to be dependent on *de novo* protein synthesis. While FGF-8 is a known androgen-induced gene in S115 cells, and its mRNA could be detected in S115 cells just prior to TSP-1 downregulation, the hypothesis that FGF-8 can mediate the effect of testosterone on TSP-1 regulation was tested. When FGF-8 was added to cells that were deprived of

serum and testosterone, a significant decrease in TSP-1 mRNA level was detected. Thus, the effect of ectopic FGF-8 mimicked the effect of Te. However, when FGF-8 action was blocked by FGF-8 antisense oligonugleotides (Mattila *et al.*, 2001a) or by a neutralizing FGF-8 antibody, Te was still able to repress TSP-1. These results led to the conclusion that FGF-8 and Te both regulate TSP-1 expression independently of each other. The role of FGF-8 in TSP-1 repression was further supported by the finding that FGF-8 overexpressing S115 cells showed significantly decreased TSP-1 levels in the presence or absence of testosterone when compared to the transfection control cell line. In addition, FGF2, another angiogenic member of FGF family, downregulated TSP-1 mRNA levels in the *in vitro* cultures of S115 cells.

5.2.2 Signaling pathway mediating FGF-8 –induced repression of TSP-1

Next, the signalling pathways leading from FGF-8 to TSP-1 repression were studied in detail. By using specific protein kinase inhibitors for MEK1/2, PI3K and p38, their involvement in TSP-1 repression was examined in cell culture. Inhibition of MEK1/2 led to the restoration of TSP-1 expression in the presence of FGF-8 in a short (10 h) treatment period. However, after 24 h of treatment, the response was only partial, even though fresh inhibitor was added after 12 h of treatment. Interestingly, when the PIK3 inhibitor, LY2940032, and the MEK1/2 inhibitor, U0126, were used simultaneously, TSP-1 expression was restored to the control level in the presence of FGF-8 at both the 10 h and 24 h time points. These results suggest that TSP-1 expression is repressed by both the MEK-ERK and PI3K pathways in response to FGF-8. A similar conclusion was made from the experiments with FGF-8 overexpressing S115 cells, in which the TSP-1 expression level was increased more by the combined inhibition of the MEK1/2 and PI3K pathways than by inhibiting only one of them at a time. Furthermore, MEK1/2 inhibition also reversed the FGF-8-mediated repression of TSP-1 in MCF10A cells, demonstrating that the mechanism is not restricted only to S115 cells.

5.3 DIFFERENTIAL FGFR SIGNALLING IN BREAST CANCER CELLS (III)

5.3.1 Silenging of FGFR1,2 and 3 in S115 cells

To study the roles of different FGFR forms in the growth of S115 cells and tumours, FGFR1,2 and 3 were subjected to shRNA-mediated gene silencing. The lentiviral transfections of the constructs targeting FGFR1, 2 and 3 resulted in cells with differential FGFR status, which we named as shR1, shR2 and shR3 cells, respectively. All puromycin resistant cells were used as a pool of shRNA expressing cells, representing a heterogenous mix of either FGFR1, 2 or 3 silenced cells. The transfections were followed by quantification of FGFR mRNA levels by quantitative RT-PCR with gene specific primers. FGFR1 was shown to be the most abundant receptor in the parental S115 cells, followed by FGFR2 and FGFR3, the latter being expressed at a relatively low level. The silencing of mRNA expression was shown to

be successful with all of the constructs: FGFR1 mRNA was reduced by 90% in shR1 cells, and FGFR2 and FGFR3 by more than 75% when compared to the transfection control shLacZ cells. However, some unexpected changes in the FGFR expression levels were observed. The cells with silenced FGFR2 and FGFR3 expression showed a 2- to 3-fold increase in their FGFR1 mRNA level. The FGFR isoforms were identified by qPCR analysis with FGFR1-2IgIIIb- and -IgIIIc-specific primers. The results showed that the sh-S115 cells almost exclusively express the IgIIIc isoform of FGFR1 and 2. Importantly, the silencing effect was shown to be effective by all of the constructs at the protein level as well, but an increased FGFR1 level was detected only in shR2 cells. Because the protein level is obviously the most important determinant in the outcome of receptor action, the FGFR expression levels of the cells refer to protein expression levels based on western blotting results from this point forward. Summary of the proportional FGFR expression in the shS115 cells and the main findings of their altered properties (described in detail below) are illustrated in Figure 6.

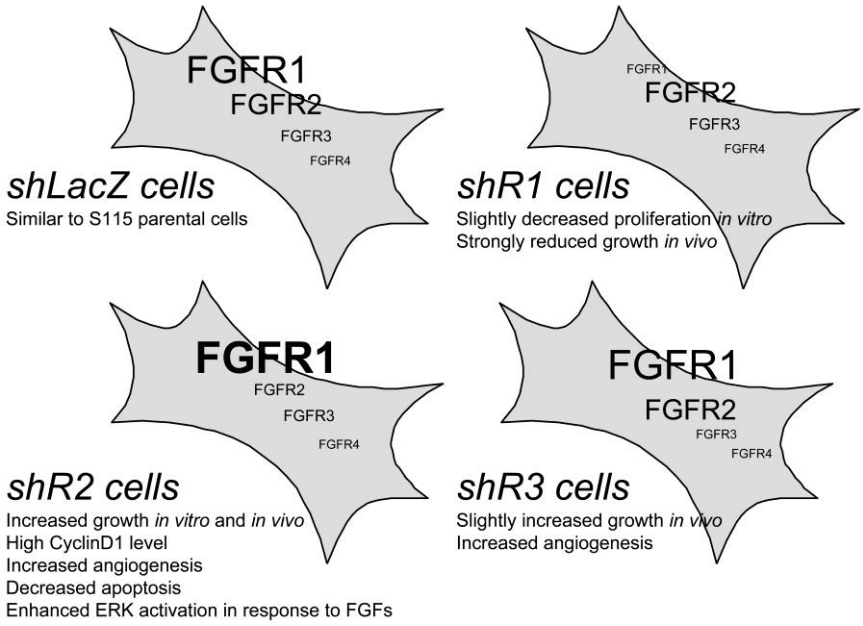

Figure 6. Summary of the altered characteristics of the shRNA expressing cells compared to the shLacZ control cells. The FGFR font size in the figure represents the approximate FGFR status of the particular pool of cells.

5.3.2 Role of FGFR1,2 and 3 in cellular proliferation in vitro and in tumour growth in vivo

The proliferation rate of shS115 cells was studied *in vitro* by means of thymidine incorporation. The results showed that shR1 cells had a decreased proliferation rate, whereas shR2 cells proliferated more rapidly than the others. shR3 cells did not differ from shLacZ cells in terms of proliferation. All the cells responded to exogenous FGF-8 by a similar increase in their proliferation rates, and the effect could be reversed with the FGFR inhibitor, PD173074. This finding suggests that all the cells have FGFR levels that are sufficient to mediate the proliferative action of FGF-8 *in vitro*. Moreover, the increased growth rate of shR2 cells was also reflected in the Cyclin D1 and B1 levels, which were significantly higher compared to the other cells, indicating a highly active cell cycle in shR2 cells.

To study the role of the different FGFRs in terms of *in vivo* growth, shS115 cells were inoculated subcutaneously into the flanks of male nude mice. The testosterone in the mice was maintained at high levels with Te pellets. The tumour growth was monitored for four weeks with callipers. Although some differences between the cell pools had already been detected in the *in vitro* proliferation assays, the differences observed in the *in vivo* growth were much more pronounced. The shR1 cells lacking FGFR1 expression did not form proper tumours, and only a few very small samples were obtained from them. shR2 cells, in turn, formed large, well-vascularised tumours. The shR3 tumours did not differ from the control shLacZ tumours in size. The experiment was repeated once, with additional groups of shR2 tumour bearing mice that were treated with PD173074 or vehicle 5 times per week for the last half of the experiment. The shR2 tumour growth was sustained when the PD173074 administration started, and the final tumours sizes decreased to the level of shLacZ tumours. To examine cell proliferation within tumours, tumour sections were immunostained for P-HisH3, which is abundant in cells undergoing mitosis. The shR3 tumours with slightly increased FGFR1 expression levels did not show an increase in the proportion of P-HisH3 positive cells compared to control tumours. The shR2 tumours showed a significantly increased level of P-HisH3 staining ($p=0.035$) compared to the control shLacZ cells. Moreover, treatment of shR2 tumour-bearing mice with the FGFR inhibitor, PD173074, led to a reduced number of proliferating cells when compared to the vehicle-treated shR2 tumours ($p=0.001$).

As the *in vivo* growth results suggested a possible growth inhibiting role for FGFR2, FGFR2IgIIIb and IgIIIc forms were transiently overexpressed in S115 cells. Overexpression of FGFR2, however, did not decrease proliferation rate or cyclin D1 protein level in the transfected cells. Quite the opposite, a strong over-expression of FGFR2IgIIIc led to slightly increased growth in *in vitro* assay, suggesting that FGFR2 at least when over-expressed, does not inhibit growth of these cells. Therefore, the growth differences between shR1 and shR2 cells are most likely the result of different FGFR1 expression levels.

5.3.3 Morphology of the sh-cell tumours

Hematoxylin and eosin (HE) stained tumours showed that silencing of different FGFRs had profound effects on the morphology of the tumours. shR1 tumours had only small areas of viable tumour cells surrounded by necrotic and fibrotic tissue, whereas the shR2 tumours were rich in capillaries and showed little necrosis. shLacZ tumours and shR3 tumours were both rich in capillaries but did contain some necrotic areas. Vascularisation in the tumours was further visualised by PECAM-1 immunostaining and quantified by counting the length of PECAM-1-positive capillaries. The capillary density in shR2 and shR3 tumours was higher than that in shLacZ tumours ($p<0.05$), whereas shR1 tumours did not show any quantifiable PECAM-1 staining. The treatment of shR2-tumour-bearing mice with PD173074 reduced the vessel density of the tumours, although the effect did not reach statistical significance.

5.3.4 Differential ERK1/2 activation in cells lacking FGFR1,2 or 3

To study how differential FGFR expression affects the FGF signal transduction, we treated the cells with exogenous FGF-8, FGF-2 and FGF-7 for 5-180 min and examined the ERK1/2 and Akt phosphorylation after the treatment. The results of western blots showed that FGF-8b and FGF-2 strongly activated the ERK/MAPK pathway in shLacZ, shR1, shR2 and shR3 cells, which was seen in increased levels of phospho-ERK1/2. However, there were some differences in the amplitude and duration of the activation. In shLacZ and shR1 cells, ERK1/2 activation peaked 5 min after FGF-8b addition and then rapidly decreased to low levels that were maintained over the 3 h time course. Except for a slightly weaker activation, ERK1/2 activation in shR3 cells was similar to that in shLacZ cells and in shR1 cells. In the shR2 cells, which overexpressed FGFR1, FGF-8b caused a two-fold higher level of ERK activity at 5 min compared to the other cells and the high level was sustained through the 3 h time course. FGF-2 treatment caused a similar pattern of ERK phosphorylation but the signal intensities were weaker in shR1 cells. FGF-7 binding FGFR IgIIIb forms caused only very small increases in P-ERK levels, which is in accordance with low proportion of IgIIIb forms of FGFRs in S115 cells. As was expected based on our previous data regarding PI3K activity in S115 cells, all of the shS115 cells showed constitutively high levels of phosphorylated Akt, and ectopic FGF-8b did not cause further increases.

5.3.5 Mechanism of FGFR1 upregulation in FGFR2 silenced cells

Since FGFR1 up-regulation was observed in FGFR2-silenced cells, we tested whether overexpression of FGFR2 would decrease FGFR1 expression. We achieved high transient overexpression of both FGFR2IgIIIb and FGFR2IgIIIc forms in shLacZ cells, but neither of these transfections had an effect on FGFR1 mRNA or protein levels. We also silenced FGFR2 using siRNA in S115 and MCF-7 cells. Despite the efficient downregulation of FGFR2 we did not detect changes in FGFR1 mRNA or protein

levels 48-72 h after FGFR2 siRNA transfection. These results suggest that FGFR1 expression is not directly regulated by FGFR2. To study whether increased FGFR1 level in shR2 cells was in any case related to FGF signalling, the cells were treated with PD173074. Interestingly, PD173074 down-regulated FGFR1 mRNA levels significantly in shLacZ, shR2 and shR3 cells. The effect was strongest in shR2 cells, suggesting that increased expression of FGFR1 in these cells is dependent on active FGF signalling. Next, the cells were deprived of testosterone and serum to decrease autocrine and paracrine signalling. In starved cells, FGFR1 mRNA levels decreased to the same level as in shLacZ, shR2 and shR3 cells, further suggesting that FGFR1 was upregulated in shR2 cells by FGF-8 or other FGFs included in the serum-containing growth medium. Because FGF-8 is the most abundantly secreted FGF in S115 cells when grown in the presence of androgens, we tested whether FGF-8b is able to increase FGFR1 expression in serum- and testosterone-starved S115 cells. After 24 h, the FGFR1 mRNA level was significantly higher in FGF-8b-treated cells than in control cells, and the effect could be blocked by PD173074. We also added FGF-8b to human MCF-7 breast cancer cells, which do not have high endogenous FGF-8 expression. Interestingly, they showed similar response to FGF-8b as S115 cells, suggesting that FGF-8b can upregulate FGFR1 in different types of breast cancer cells.

5.3.6 Regulation of FGF-8 target genes in cells lacking FGFR1,2 or 3

To clarify the possible differences in FGF-8 target gene regulation in the shS115 cells, we studied TSP-1 and OPN mRNA expression in FGF-8-stimulated sh-cells by quantitative PCR method. The result showed a similar response after FGF-8 treatment in all the cells. Specifically, all of them showed a marked downregulation of TSP-1 and upregulation of OPN in the presence of FGF-8. These results show that regardless of the altered FGFR levels, all of the sh-cells have a sufficient level of FGFRs to mediate FGF-8 action, as shown by the FGF-8-induced proliferation of these cells.

6 DISCUSSION

Most of the observations reported in this thesis study were obtained by using the S115 model of breast carcinoma. The S115 cell line was originally derived from a spontaneous adenocarcinoma of the mammary gland of a female DD/Sio mouse. In spite of its origin in the mammary gland, in serial transplantation experiments the tumours were found to grow only in male mice and to be androgen regulated (Minesita and Yamaguchi, 1965). The S115 cell line was then characterised by numerous studies as a model for steroid-regulated malignant growth (Smith and King, 1972; Darbre and King, 1988; Harkonen *et al.*, 1990). Moreover, our studies and those of others have shown it to represent a model system for FGF-dependent malignant transformation (Tanaka *et al.*, 1992; Kouhara *et al.*, 1994; Ruohola *et al.*, 1995; Mattila *et al.*, 2001b). It is apparent that while being an androgen regulated mouse cell line, S115 model is not the most appropriate model for studying breast cancer, and we are aware of the challenges that exist when interpreting the data obtained from these cells. However, in the light of the new information concerning breast cancer subtypes (discussed in 2.1.2), it is interesting to note that according their steroid receptor status, S115 cells represent cells with some similar features to tumour cells from AR-positive TNBCs (Gucalp and Traina, 2010). Thus valuable observations regarding AR and FGF signalling and the relationship between them may result from studying S115 cells. This type of investigation may lead to a better understanding of the mechanisms that may also be involved in the growth of the challenging triple-negative form of breast cancer.

6.1 REGULATION OF TSP-1

Thrombospondin-1 (TSP-1) is a multifunctional ECM protein, which has been indicated to have an important role in the tumour angiogenesis and metastasis. In the S115 model, we detected a strong repressive effect of testosterone (Te) on the TSP-1 mRNA and protein levels. We were able to show that Te repression of TSP-1 was dependent on *de novo* protein synthesis but that the effect was independent of FGF-8 induction. However, blocking FGF-8 action in the presence of Te did reduce the effect of Te to some extent, suggesting an additive role for FGF-8 in the Te-induced TSP-1 repression. The very first report concerning androgen regulation of TSP-1 expression was published a few years ago (Colombel *et al.*, 2005). In this particular study, TSP-1 expression was shown to be decreased by androgens in rat ventral prostate and in prostate cancer patients after testosterone deprivation treatment. According to a gene reporter assay in SaOS-2 osteosarcoma cells, the regulation was proposed to be mediated through a putative ARE in the TSP-1 promoter. In addition, androgens are shown to repress TSP-1 in a model for bladder cancer (Johnson *et al.*, 2008). However, it remains unconfirmed if TSP-1 is a direct target gene for activated AR in cancer cells. In addition to androgens, TSP-1 has been shown to be under the regulation of other sex steroids. However, there is some controversy in the data concerning oestrogen regulation of TSP-1. Oestrogens were first shown to repress TSP-1 expression in breast

cancer cells (Sengupta *et al.*, 2004), but quite recently, E2 was shown to induce its expression in two different luminal breast cancer cell lines (MCF-7 and T47D) in an ERα-dependent manner (Hyder *et al.*, 2009a). The same authors also studied progesterone regulation of TSP-1 and found that natural and synthetic progestins induce TSP-1 expression in T47D cells in a PR-dependent manner (Hyder *et al.*, 2009b). Furthermore, they suggested that the induction of TSP-1 is important in promoting proliferation and angiogenesis in breast cancer. In contrast, TSP-1 expression is silenced in several breast cancer cell lines, which has been demonstrated to have a central role in the angiogenic switch of breast cancer cells (Watnick *et al.*, 2003).

TSP-1 is under the regulation of several growth factor pathways, oncogenes and tumour suppressors, such as p53, p76, PTEN, Ras and Myc (Dameron *et al.*, 1994; Tikhonenko *et al.*, 1996; Rak *et al.*, 2000; Vikhanskaya *et al.*, 2001; Wen *et al.*, 2001). In the present study, we found that TSP-1 is downregulated in S115 cells by FGF-8 through the activation of ERK and PI3K pathways, both of which have been previously reported to be involved in TSP-1 repression (Watnick *et al.*, 2003; Ridnour *et al.*, 2005). Interestingly, p38 inhibition in our study led to decreased TSP-1 expression, suggesting that p38 activity is crucial to maintaining the high basal TSP-1 expression in S115 cells. The association between p38 activity and increased TSP-1 expression has also been reported previously (McGillicuddy *et al.*, 2006; Zhao *et al.*, 2008). Taken together, the ERK1/2, PI3K and p38 pathways seem to play opposing roles in the regulation of TSP-1 mRNA expression in S115 cells. Because the ERK1/2 and PI3K pathways are more active in S115 cells than the p38 pathway, FGF-8 stimulation leads to an overall reduction in TSP-1 mRNA levels. A link between FGF family members and TSP-1 expression was suggested already 15 years ago, when TSP-1 was recognised as an FGF-1 and FGF-2 target gene in endothelial cells (Ashton *et al.*, 1995). Our study is, however, the first to report a regulatory relationship between FGFs and TSP-1 in cancer cells. Taken together, our results suggest that the repression of TSP-1 could be one of the mechanisms responsible for the angiogenic properties of FGFs. The best known antiangiogenic actions of TSP-1 involve the inhibition of endothelial cell proliferation and migration and the induction of apoptosis in these cells (Armstrong and Bornstein, 2003), but it is clear that TSP-1 functioning in the tumour microenvironment has numerous other effects as well. Interestingly, FGFs and TSP-1 interact in the ECM, and TSP-1 has been suggested to have an impact on ECM-associated FGFs by affecting their location, bioavailability and function (Presta *et al.*, 2005). Thus, the antiangiogenic properties of TSP-1 could also result, at least partly from its capacity to bind angiogenic FGF-2 and through the generation of inactive TSP-1/FGF-2 complexes (Taraboletti *et al.*, 1997; Margosio *et al.*, 2003). By repressing TSP-1 production, FGF-2 and FGF-8 may potentiate their own paracrine and autocrine action by increasing their concentration in ECM and binding to FGFRs. However, the details of the transcriptional regulation at the TSP-1 promoter by FGF signaling remain to be studied. Among the numerous putative transcription factors binding sites in the mouse TSP-1 promoter, for example, several Ets sites might be targets for FGF-8-induced transcriptional repression of TSP-1 because Ets family

transcription factors are known to mediate the transcriptional effects of FGFs (Acevedo *et al.*, 2009).

The seemingly opposite reports on TSP-1 expression and its tumour growth-promoting versus antitumourigenic effects obtained from the different breast cancer cell lines reflect very well the overall complexity of the role of TSP-1 during cancer progression. An interesting result was obtained in a study conducted in a Pyt transgenic mouse model, where TSP-1 was shown to inhibit angiogenesis and tumour growth of the primary tumours but concomitantly promote metastasis to the lung (Yee *et al.*, 2009). In the light of recently raised concerns regarding angiogenesis inhibitors that seem to promote metastasis, at least in experimental models (described in section 2.3), the diverse effects of TSP-1 might result from the same phenomenon, where inhibiting angiogenesis, even by an endogenous factor, may act as driving force for a more invasive and metastatic phenotype (Paez-Ribes *et al.*, 2009). It is interesting that the plasma TSP-1 levels were found to be higher in advanced breast cancer patients compared to early stage breast cancer patients and that levels in both of these groups are higher than in healthy controls (Byrne *et al.*, 2007).

As discussed above, it is evident that TSP-1 is under the regulation of several hormones and growth factor pathways, and it is likely that many of the contradictory results can be explained by the context-dependent regulatory pathways and by the multifunctional nature of the TSP-1 protein itself. Altogether, there is good evidence that TSP-1 inhibits primary tumour growth but that it may also have different and growth stimulatory effects depending on the cellular context and hormonal milieu.

6.2 ANDROGENS AND THE REGULATION OF ANGIOGENIC FACTORS IN THE S115 MODEL

Much is known about the association of androgens and angiogenesis in the rodent prostate, where androgen withdrawal induces endothelial cell apoptosis and vascular regression, which is in turn preceded by epithelial cell apoptosis (Franck-Lissbrant *et al.*, 1998; Jain *et al.*, 1998). In the S115 carcinoma model, androgens have been shown to induce angiogenesis and maintain the tumour vasculature (Takatsuka *et al.*, 1992; Jain *et al.*, 1998). Androgen deprivation leads to decreased VEGF mRNA and protein expression in LnCap cells (Stewart *et al.*, 2001) and in androgen-responsive human prostatic cancer xenograft models (Joseph and Isaacs, 1997). In addition, the castration of mice bearing LnCap tumours results in a rapid reduction in VEGF mRNA expression and markedly reduced tumour neovascularisation (Stewart *et al.*, 2001). Furthermore, androgens have been shown to increase VEGF secretion in many studies, including studies in LnCap and S115 cells (Joseph and Isaacs, 1997; Ruohola *et al.*, 1999). Thus, the regulation of VEGF secretion is proposed to be an important mechanism behind androgen-regulated angiogenesis. Our results further suggest that the effects of androgens on tumour angiogenesis, at least in the S115 model, are mediated by combined effects on the expression of pro- (FGF-8, VEGF) and

antiangiogenic (TSP-1) factors, and that by repressing TSP-1, androgen-induced FGF-8 potentiates the effect of Te. A summary of the regulation of angiogenic factors in the S115 model based on the results obtained in the thesis study and the previous data on VEGF regulation is presented in Figure 7.

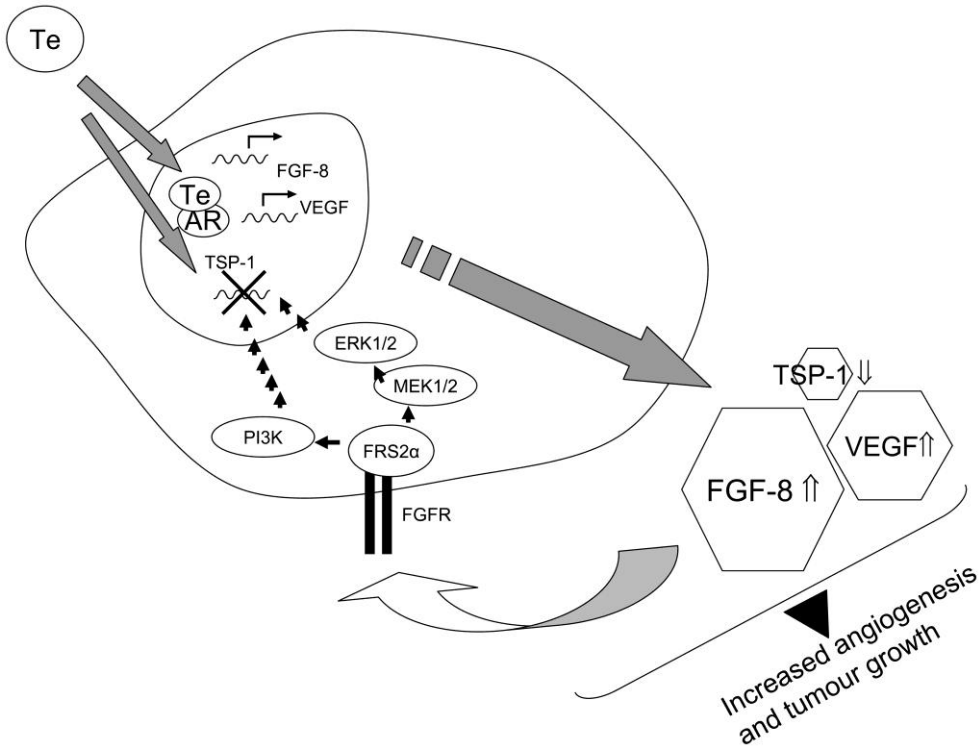

Figure 7. Summary of the regulation of S115 cell and tumour growth and angiogenesis (Ruohola *et al.*, 1999, results from this thesis)

6.3 FGFs AND FGFRs IN BREAST CANCER CELLS

Aberrant regulation or function of the FGF-FGFR signalling system has been implicated in the development and progression of mammary tumours in murine experimental models and in human breast cancers, as described in the review of the literature (2.2). Genetic alterations and/or the over-expression of FGFR1, 2 and 4 have been reported in human breast cancer (Table II), and there is increasing evidence that FGFR signalling is involved in the development of drug resistance in certain types of breast cancers (Roidl *et al.*, 2009; Marme *et al.*, 2010a; Turner *et al.*, 2010b). The differential signalling of FGFRs in breast- and prostate-derived cells has been previously characterised by drug-inducible systems, in which FGFR1 or 2 signalling

can be triggered by synthetic compounds independently of endogenous FGFs or FGFRs (Welm *et al.*, 2002; Freeman *et al.*, 2003; Xian *et al.*, 2005; Xian *et al.*, 2007; Xian *et al.*, 2009). In those studies, it was shown that FGFR1 activation drives a more malignant phenotype that includes EMT, increased proliferation and invasiveness and is associated with strong and sustained ERK activity, whereas FGFR2 activation leads to apoptosis and only a transiently activated ERK pathway and receptor downregulation.

We chose to use a different strategy to study the differential role of these receptors and focused on silencing the endogenous FGFRs. We used shRNA constructs with stable lentiviral transfections to achieve new S115 cell pools representing differential FGFR expression status. As a result, we successfully silenced the expression of FGFR1, -2 and -3 mRNA and protein expression. Interestingly, we observed a significantly increased FGFR1 expression in cells that had been subjected to FGFR2 silencing. It was previously shown that FGF-2 increases FGFR1 expression in S115 (Ruohola *et al.*, 1995). Very recently, FGF-8 was also reported to increase FGFR1 expression in mouse 3T3 fibroblasts and GT-17 neuronal cells (Mott *et al.*, 2010). Thus, it could be speculated that FGFR2 silencing could allow enhanced FGF-8 mediated autoregulation of FGFR1, leading to significantly increased FGFR1 expression in S115 cells. Indeed, the upregulation of FGFR1 was dependent on FGF signalling, and it could be reversed by either inhibiting FGF signalling or by depriving the cells of serum and growth factors. Furthermore, we showed that FGF-8b increased FGFR1 expression, not only in S115 cells, but also in human MCF-7 breast cancer cells. In contrast, the overexpression of FGFR2 had no effect on FGFR1 levels, suggesting that the regulation is mediated via FGFR1 itself. Taken together, these results show that FGFR1 is subjected to regulation by FGF-8 (and probably also by other FGFs). The magnitude of FGFR1 autoregulation seems to be related to the cellular level of FGFR2 (and possibly FGFR3), and the altered balance between FGFRs but the mechanisms involved remain to be explored. FGFR1 upregulation may also result from the activation of compensatory pathways triggered by the downregulation of FGFR2. Transcriptional activation of the FGFR1 gene has already been shown to be activated by the Sp1 and E2F transcription factor pathways (Tashiro *et al.*, 2003; Seyed and Dimario, 2007; Kanai *et al.*, 2009).

In any case, the overall conclusion based on our studies is, that FGF signalling is indeed crucial for the growth of tumours originating from the FGFR silenced cells. The high FGFR1 expression in shR2 cells was shown to provide the cells with a strong proliferative capacity *in vitro* and *in vivo,* and moreover, its expression was a prerequisite for the *in vivo* growth of S115 cells. The cells with silenced FGFR1 but unchanged expression of FGFR2 proliferated at only a slightly slower rate *in vitro,* but in contrast to all other sh-cell lines, they did not form proper tumours *in vivo*. Thus, the impact of silencing FGFRs on the growth rate was much more pronounced *in vivo* than *in vitro*, which highlights the important role of the tumour environment. However, the transient FGFR2 overexpression in S115 cells promoted rather than inhibited proliferation *in vitro*; thus, it is likely that the major differences in the growth rates of

the cells with different relative levels of FGFR1 and FGFR2 expression are due to a dose response effect of FGFR1 expression.

In addition to proliferation, we also studied angiogenesis and cell death in sh-cell tumours. The density of capillaries in shR2 and shR3 tumours was higher than that in basically angiogenic shLacZ control tumours, whereas in the slowly growing shR1 tumours capillaries were very sparse and not quantifiable. The result suggests that the angiogenic capacity of shR2 tumours is mediated by FGFR1, which may partially explain the dramatically reduced growth rate of shR1 tumours lacking FGFR1 expression. In addition, there may be a role for non-canonical FGFR signalling which results in FGFR mediated responses independently of FGF ligands (Murakami *et al.*, 2008; Francavilla *et al.*, 2009). For example, neural CAM (NCAM) was recently shown to induce sustained FGFR1 activation through receptor recycling and stabilisation, leading to the stimulation of signalling cascades distinct from those induced by FGF (Francavilla *et al.*, 2009). This interaction between NCAM and FGFR1 in the tumour microenvironment could possibly potentiate the effects of FGFR1 expression and explain the slow growth in the absence of FGFR1. The tumours originating from cells with silenced FGFR2 and high FGFR1 expression (shR2 cells) showed a reduced number of apoptotic cells when compared to other tumours. FGFR1 silencing in FGFR2-expressing cells (shR1 cells) in turn did not influence the frequency of apoptotic cells, suggesting that FGFR1 expression is not a prerequisite for the survival of S115 cells. The results also suggest that if FGFR2 is the only FGFR present, it is sufficient to protect the cells from apoptosis. Recently, it was shown that FGFR-mediated PI3K activity is crucial to prevent apoptosis in 4T1 breast cancer cells (Dey *et al.*, 2010). Similarly, FGF-8b protected S115 cells from apoptosis via PI3K (Nilsson *et al.*, 2009). However, as the studies of this thesis show, PI3K activity remains high in S115 cells under serum starvation conditions, and we could not detect any difference in Akt phosphorylation between the FGFR-silenced cell lines in the presence or absence of exogenous FGF-8b. Thus, either the remaining FGFRs together with auto/paracrine FGFs are sufficient for continuous PI3K activation in all of the shRNA expressing cells or PI3K is also activated by mechanisms other than those mediated by FGFRs in S115 cells. In either case, the sustained PI3K activity could explain the relatively low apoptotic index in all tumours.

In our study, the shR2 cells expressing high FGFR1 and low FGFR2 levels responded to exogenous FGF-8b and FGF-2, as evidenced by stronger and more sustained ERK1/2 activation than in the cell lines expressing normal levels of FGFR2. This result may partially explain the differences in growth rates between the cell lines, as ERK activation downstream of FGFRs has been associated with proliferative responses (Dailey *et al.*, 2005). Importantly, our results further suggest that FGFR1 plays a major role in FGF-driven ERK activation, leading to the increased growth and survival of cancer cells. However, ERK was also phosphorylated in response to FGF-8b and FGF-2 in FGFR1-silenced shR1 cells with relatively low overall FGFR expression status, which was almost exclusively comprised of FGFR2 expression. This

finding suggests that the activation of ERK by FGFR2 also mediates outcomes other than proliferative responses.

Treating the mice bearing shR2 tumours with the FGFR inhibitor, PD173074, reversed the changes in capillary density, proliferation and angiogenesis to the level of control shLacZ tumours, suggesting that high FGFR1 signalling in shR2 tumours plays a crucial role in all of these responses. However, because PD173074 also inhibits the VEGFR and PDGFR tyrosine kinases (Hynes and Dey, 2010), the effects obtained by PD173074 treatment may not be solely related to FGFR blockage. Interestingly, FGFR inhibition by another FGFR inhibitor, TKI258, led to similar effects regarding vessel density, proliferation and apoptosis in 4T1 and 67N mammary tumour cells *in vivo* as achieved here by PD173074, and these effects were shown to be due to FGFR inhibition (Dey *et al.*, 2010).

Taken together, our results regarding the differential role of FGFRs in the growth of S115 breast cancer cells are in accordance with the previous literature concerning differences between FGFR signalling in breast and in prostate cancer cells. Thus, the growth-promoting effects of FGFR1 are prominent, emphasising the important role of FGFR1 activity in breast cancer growth and progression. FGFR2, in turn, may mediate growth inhibitory signals in breast tumourigenesis; however, with the exception of the weaker ERK activation, the mechanisms remain to be determined. Considering the future promise of FGFR modulators as possible therapeutic agents in human cancers, revealing the different responses to FGFR1-4 activation is of high importance.

7 CONCLUSIONS

1) Androgens repress TSP-1 expression in breast cancer cells. The repression requires *de novo* protein synthesis in S115 cells. However, direct repression due to an ARE in the TSP-1 promoter has also been reported by others. In addition, androgens can additionally repress TSP-1 via the induction of FGF-8. Together with the existing literature, the results of this thesis strongly suggest that androgens (and FGF-8 by mediating androgen effects) may be important contributors in regulating the balance of the angiogenic factors and, thus, the angiogenic switch in the tumour microenvironment of the breast cancers with active AR (e.g., TNBC).

2) Repression of TSP-1 by activation of MEK-ERK and PI3K pathways is probably one of the main mechanisms that explain the angiogenic properties of FGF-8 and the other angiogenic FGF family members. Decreased TSP-1 production contributes to the altered balance of angiogenic factors during the angiogenic switch, which in turn is suggested to lead to the uncontrolled endothelial cell proliferation that occurs during tumour neovascularisation.

3) The proportions of different FGFRs in breast cancer cells are highly important in determining the outcome of FGFR activation: FGFR1 signalling is crucial for breast cancer cell growth *in vitro* and *in vivo*, whereas FGFR2 alone is unable to support tumour growth *in vivo*. Different FGFRs may induce ERK activation and may also induce other intracellular signalling cascades at different strengths and amplitudes. These effects are known to be fundamental in determining the response to FGF stimulus.

4) This study further support the previous evidence that FGF-FGFR signalling contributes to breast cancer cell growth in several ways, including the promotion of proliferation and angiogenesis and by reducing apoptosis in tumours. Thus, the recently discovered genetic alterations leading to the altered expression or activity of several FGFR forms can give a significant growth advantage to cancer cells and contribute to the progression of advanced disease and to hormone-resistant growth.

5) Based on the accumulating data on FGFR signalling in breast cancer, the FGF/FGFR system provides potential targets for the development of novel therapies in human breast cancer. However, as this thesis study and the previous literature suggest, different FGFRs may have differential effects in the tumour microenvironment. More studies on these differences and the importance of the FGFR status of the cells are highly warranted for identifying those patients that could benefit from interfering with this pathway in the future.

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